The Effect of Indoor Management Systems for Pregnant Ewes on Maternal Behaviour Expressed After Parturition

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Declaration

I hereby declare that this thesis has been composed by me and the work is my own, except as acknowledged by means of references. This thesis has not been submitted for any other degree or professional qualification except as specified.

Nur Nadiah Md Yusof

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

List of abbreviations frequently used throughout this thesis:

Abbreviation	Definition
BCS	Body Condition Score
BHOB	Beta-hydroxybutyrate
CI	Confidence Interval
FGM	Faecal Glucocorticoids Metabolite
HPV	High-pitched vocalisation
IgG	Immunoglobulin G
L1	Lamb 1
L2	Lamb 2
LPV	Low-pitched vocalisation
SEM	Standard Error of the Mean

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Lay Summary

Maternal behaviour displayed by the ewe towards its lamb is important in improving the survival of newborn lambs. To be able to recognize and nurture its own lamb, ewes lick and groom the newborn lamb and cooperate with sucking attempts as well as producing a maternal rumbling vocalisation or low-pitched bleat, directed to her own lamb. However, maternal behaviour may be affected by a number of factors which include the social environment and housing management on the farm where sheep are kept. In the UK, most sheep are farmed outdoors in extensive systems although it is a normal practice to house ewes during winter until lambing time to protect the ewes and lambs from adverse environmental conditions. In the indoor housing system, ewes may be subjected to small space allowance, mixing with unfamiliar ewes, provided with different type of diet as well as being frequently handled by humans. Therefore, the purpose of this study is to assess whether indoor housing management may cause stress to the pregnant ewes and impair the maternal behaviour displayed towards their newborn lamb after lambing. The assessment will be based on ewe condition, behaviour, and physiological indicators recorded in the ewes during pregnancy and after lambing. In this study, the experience of the ewes in giving birth (inexperienced or lambed previously) and the temperament of the ewes were also considered in the assessment.

In the first study, 72 ewes were divided into two groups: Control and RS-Mix (Restricted-Space and Mixed). RS-Mix ewes were provided half the amount of pen and feeding trough space given to the Control ewes. RS-Mix ewes were also regrouped with other ewes 3 times during gestation. Higher aggression in RS-Mix ewes was only seen during the first week of observation which suggested that the sheep may have adapted to the conditions. However, after lambing, RS-Mix ewes were less cooperative with their lambs when they tried to suck and primiparous ewes from this group took a longer time to approach their own lambs in recognition test, suggesting some impacts of the housing on maternal behaviour. Inexperienced ewes were more affected than experienced ewes, with higher concentration of stress hormones and the lowest weight gain throughout the experiment. During the first study it was observed that Control ewes also showed aggression at feeding. Therefore, in the second study, an alternative

housing system was designed to reduce competition and aggression at feeding, by allowing continual access to feed. The space allowance in this system (Alternative) was more than three times that of the other ewes, and they were provided with ad libitum grass silage and left undisturbed until lambing.

The Alternative system did reduce aggression but also caused loss of body condition and low weight gain, as well as the highest concentration of stress hormones during gestation. After lambing, the Alternative ewes were slower to begin grooming the lamb, spent the least time grooming their lambs, and were less cooperative when the lambs attempted to suck. These outcomes suggested the Alternative feeding system was not providing the ewes with their full requirements, perhaps because the ewes did not find the feed palatable. Inexperienced ewes also were less cooperative in assisting with lamb sucking attempts as they may have more difficulty in adjusting to the new environment than older and more experienced ewes which contribute to greater stress in this group.

In conclusion, even though the behaviour and physiological parameters recorded during gestation were insufficient to verify the existence of stress, the alteration of certain aspects in maternal behaviour recorded after parturition may have confirmed that management system in indoor housing could cause stress to the ewes. However, an altered feeding system with more space per animal, designed to reduce aggression between animals, was also shown to impair maternal behaviour in ewes, probably due to undernutrition in these animals. Therefore, special attention should be taken to provide the ewes with adequate housing condition and fulfil the nutritional requirements of gestating ewes in order to ensure the establishment of stronger ewe-lamb interaction which will improve the survivability of newborn lambs.

Abstract

The survival of newborn lambs is highly dependent on the successful partnership between ewes and their offspring. High lamb mortality before one week of age is largely due to hypothermia, mismothering and starvation, which are indirectly consequences of poor establishment of a maternal bond between a ewe and her lambs. Maternal behaviour displayed by ewes towards their lambs is therefore crucial in ensuring the survival of the newborn lamb. However, maternal behaviour can be affected by many factors including the social environment, nutrition and husbandry routines. It is common in the UK for sheep to be kept indoors during winter from mid gestation until lambing to protect ewes and lambs from adverse conditions. Keeping the ewes indoor nonetheless, has its own challenges as the animals may have to be mixed with unfamiliar conspecifics, have limited floor and feeding space, changes to the diet and increased handling by humans. Therefore, the main objective of this study was to investigate the effect of housing systems experienced by ewes during gestation on mother-offspring interactions after parturition. The impact of different housing conditions were assessed by recording body weight, body condition score (BCS), behaviour, faecal glucocorticoid metabolite (FGM) concentrations and haematology parameters. After parturition, maternal behaviour was then assessed. Parity and the temperament of the ewes were also taken into account in assessing all parameters during gestation and after parturition.

In the first experiment the effect of an indoor housing system on the behaviour and physiology of 41 primiparous and 36 multiparous ewes from 11 until 18 weeks of gestation was assessed. The ewes were divided into two groups: Control and RS-Mix (Restricted-Space and Mixed) where the RS-Mix ewes were allocated half the amount of space and feedface allowance given to Control group and were also subjected to three social mixing events. No significant treatment effects on physiology or behaviour during gestation were found except for higher aggression in RS-Mix ewes during the first week of observation which gradually declined to the same level as Control ewes at the end of experiment. RS-Mix ewes also displayed significantly higher ruminating behaviour at week 18 of gestation compared to Control ewes. However, primiparous

ewes were more affected than multiparous ewes regardless of treatment group, with lower weight gain and higher FGM concentrations compared to multiparous ewes. In this study, the difference in housing system did not seem to have a lasting impact on the physiology and behaviour of pregnant ewes.

The maternal behaviour of these ewes during parturition and lactation was investigated. RS-Mix ewes were significantly more likely to give birth to Lamb 2 (L2) while standing, compared to Control ewes and also more likely to give birth during the day whereas Control ewes were equally likely to give birth during the day or night. Grooming behaviour of these ewes was not affected by pregnancy treatment but RS-Mix ewes displayed significantly higher avoidance when the lambs reached the udder to suck compared to Controls. During tests of lamb recognition, primiparous ewes from RS-Mix group took longer to approach their own lambs and during lactation on the field, they kept the greatest distance from their neighbouring ewes, compared to all other groups. This suggests that maternal behaviour in RS-Mix ewes may have been affected by the stress experienced from the space restriction, regrouping and remixing during pregnancy.

In the first experiment Control ewes as well as RS-Mix ewes displayed aggressive behaviour at the feedface during concentrate feeding. This implies that Control ewes may also experience competition at feeding which might explain the few behavioural effects of treatment. Therefore, in the next study two new housing treatments were set up: Alternative and Negative. Alternative ewes were provided with a larger space allowance of $4.57m^2$/ewe and 66cm/ewe of feedface allowance and had *ad libitum* access to food (grass silage) throughout the experiment. Negative ewes were provided with the same space and feedface allowance ($1.28m^2$/ewe and 33cm/ewe respectively) as Control ewes from the first experiment but they were subjected to additional stressors, such as exposure to dogs and delayed feeding. The Control group in this experiment was as the RS-Mix group from the previous study but without the occurrence of social mixing. Surprisingly, Alternative ewes were found to be the most affected by the treatment. The BCS of Alternative ewes decreased significantly from week 15 to week 17 of gestation and this group had the least weight gain throughout gestation compared to Control and Negative ewes. FGM concentration in Alternative ewes was also the highest compared to other treatment groups from week 13 until week 17 of gestation, and at 12 hours postpartum. Interestingly however, the concentrations of beta-hydroxybutyrate and neutrophil-lymphocyte ratio in Alternative ewes were significantly lower than other groups. Alternative ewes also took a longer time to groom their lambs, spent less time grooming and were less cooperative with the lambs sucking attempts than other groups.

This study found only minor evidence that housing systems, which mimicked commercial conditions, caused significant stress in pregnant ewes, although short-term aggression at feeding was observed in both Control and Negative groups. An alternative system, which was designed to reduce competition and aggression at feeding, by allowing continual access to feed, did reduce aggression but had other effects on the ewes, such as loss of body condition, which suggested this feeding system was not providing the ewes with their full requirements, perhaps because the ewes did not find the feed palatable. It was apparent, however, that primiparous ewes may have more difficulties in adjusting to the new environment than older and more experienced ewes, and this may contribute to greater stress in this group. Even though the behaviour and physiological parameters recorded during gestation were insufficient to verify the existence of stress, the alteration of certain aspects of maternal behaviour recorded after parturition suggested that management systems in indoor housing could cause stress to the ewes. Exposure to undernutrition during pregnancy was also shown to affect maternal behaviour in ewes. Therefore, special attention should be taken to provide the ewes with adequate housing condition and fulfil the nutrition requirements of gestating ewes in order to ensure the establishment of stronger ewe-lamb interaction which will eventually improve the survivability of newborn lambs.

1. General Introduction

1.1 Lamb mortality

Lamb mortality is a significant welfare issue affecting the efficiency and profitability of sheep production (Binns et al., 2013; Matheson et al., 2012). It is estimated that between 10 and 30% of lambs worldwide die before weaning with the highest mortality rates occurring within the first 3 days of postnatal life (Binns et al., 2002; Brien et al., 2010; Holmoy et al., 2014; Nowak et al. 2000; Sawalha et al., 2007). Dystocia, which is difficulty or abnormality in the process of giving birth, represents one of the major causes of lamb mortality (Dalton et al., 1980; Dwyer, 2008a; Dwyer & Bunger, 2012). This commonly happens in lambs selected for high production efficiency reared in extensive systems with reduced supervision (Dwyer & Bunger, 2012; Nowak & Poindron, 2006). However, from immediately after birth until approximately 1 week of age, most lamb mortality is due to hypothermia, mismothering and starvation (Chaarani et al., 1991; Dwyer and Lawrence, 1998; Green and Morgan, 1993; Mellor and Stafford, 2004; Nowak and Poindron, 2006; Yapi et al., 1990). The mortality caused by mismothering-starvation complex as well as hypothermia are affected by various factors such as adverse weather conditions, problems with thermoregulation, inadequate colostrum intake and impaired mother or lamb behaviour (Kuchel & Lindsay, 1999; Nowak & Poindron, 2006).

The survival of newborn lambs depends fundamentally on the successful partnership between mother and offspring as well as the quality of the appropriate behaviours and interactions between them (Everett-Hincks et al., 2005; Haughey, 1991; Madani et al., 2013; Matheson et al., 2012). Maternal care displayed by the ewe is important in substantially reducing lamb mortality, by a range of strategies which involve providing the offspring with nutrition, thermoregulation, immunological and physical protection, comfort, and opportunities for social learning (Dwyer, 2014; Dwyer & Lawrence, 2005). As many sheep farmers currently are interested in practicing lower input systems, compared to highly intensive systems, for both economic and social reasons (Dwyer, 2008a), the role played by maternal behaviour is crucial since the ewes must be able to conceive, carry, give birth and care for their young with relatively little human intervention (Conington et al., 2010).

1.2 Maternal Behaviour in Sheep

Maternal behaviour displayed by the ewe towards her lamb is one of the essential factors in ensuring the survival of the newborn lamb (Nowak et al., 2000). Prior to birth, ewes show no interest and even actively avoid a newborn lamb (Dwyer, 2008a, 2014). However, at parturition, which is the most critical time in the ewe's reproductive cycle, the behaviour of the ewes will change entirely by showing nurturing behaviours towards the lamb to permit the establishment of exclusive attachment between the ewe and her progeny (Dwyer & Lawrence, 2005; Poindron et al., 2007). These behaviours include high levels of licking and grooming (Alexander, 1988), frequent low-pitched bleats (specific maternal vocalisation) (Dwyer et al., 1998), and cooperation with lamb attempts to suck by standing still (Dwyer, 2008a; Dwyer & Lawrence, 2005). In addition, absence of aggression and lamb rejection, along with the maintenance of a close spatial proximity between ewe and lamb are also a part of maternal behavioural traits associated with lamb survival Dwyer & Lawrence, 2005).

All of the behaviours mentioned above serve two main functions: first, the expression of nurturing behaviour to facilitate the transition from prenatal to postnatal life as well as to promote rapid sucking (Dwyer & Lawrence, 2005), and second, to allow the ewe to recognize her own lambs so she can form an exclusive selective attachment only to her own offspring (Lévy et al., 1995). This selectivity is important to avoid the ewe nursing non-offspring which will affect the survival of her own lambs as it is unlikely that the ewe will produce sufficient milk to feed several lambs (Dwyer & Lawrence, 2005).

For maternal behaviour to commence at birth there is a sequence of events associated with late pregnancy and parturition that needs to occur beforehand. Failure of one of these events to take place will not bring about maternal responsiveness by the ewe towards her lambs (Dwyer, 2014). The first event is the decline in progesterone concentration and an increase in circulating oestradiol from the placenta during late gestation (Kendrick & Keverne, 1991). Oestradiol will then bind to oestrogen receptors and stimulate an increase in oxytocin receptors (OTR) in several areas of the brain associated with the display of maternal behaviour (Broad et al., 1999). This is then followed by vaginocervical stimulation (VCS), which is the stretching of the birth canal at delivery resulting in the release of central oxytocin from the paraventricular nucleus (PVN) of the hypothalamus (Dacosta et al., 1996), and finally, sensory cues provided by the amniotic fluids in which the birth coat of newborn lamb is soaked (Poindron et al., 2010, 2007). Besides contributing to the onset of maternal behaviour, the presence of the amniotic fluid also provides the basis of selectivity of the ewe for her own offspring by which the ewe rapidly learns the olfactory signature of her own lamb and therefore can prevent the attempts of non-offspring to suckle (Poindron et al., 2007).

1.3 Individual variation in the expression of maternal behaviour of sheep

As has been discussed previously, the commencement of maternal behaviour at birth is highly dependent on a series of events occurring at late pregnancy and parturition (Dwyer, 2014). Once the entire events in the series have successfully taken place, mother-offspring bonding will be established. However, maternal behaviour is known to be affected or impaired by several factors. Among the impairments in maternal care that have been observed in ewes are delays in displaying maternal behaviour, acceptance of only one lamb from a pair of twins, aggression towards their lambs or a complete absence of maternal responsiveness. The different factors known to influence the expression of maternal behaviour will be considered in this section.

1.3.1 Breed

One of the factors which contribute to the variation in the expression of maternal behaviour is ewe breed. There have been many studies conducted in comparing the differences in maternal behaviour expressed by different breeds of sheep. For example, on grooming behaviour, Blackface ewes (hill breed) were quicker to begin grooming their lambs after parturition and groom their lambs more compared to Suffolk ewes which is the lowland breed selected for lean tissue growth (Dwyer and Lawrence, 1998; Dwyer and Smith, 2008; Pickup and Dwyer, 2011). Blackface ewes were also observed to cooperate more when the lambs reached the udder attempting to suck (Pickup & Dwyer, 2011) and made more low-pitched vocalisations than Suffolk ewes (Dwyer & Smith, 2008). From the studies performed in the UK, it can be concluded that intensively managed lowland ewe breeds which were selected for lean tissue growth (Suffolk) tend to show poorer maternal care at birth compare to hill breeds which are usually managed extensively (Dwyer & Lawrence, 2005; Rooke et al., 2010). While studies conducted on French purebreds Romanov and Lacaune as well as crosses between them showed that Romanov (highly prolific breed) ewes spend more time licking their lambs than the Lacaunes (intensive dairy) and they were also very distraught after the removal of their lamb (Le Neindre et al., 1998). In addition, a study on three breeds of Southern Mediterranean breed sheep shows that Cukurova breed, a breed created to improve litter size, provides its lamb with more licking and grooming than Cukurova Assaf and Cukurova Meat Sheep which were created to improve milk production and meat production respectively (Ocak et al., 2013).

The concentration of circulating oestradiol has been demonstrated to effect the expression of maternal behaviour in ewes. Higher level of oestradiol concentration is positively correlated with the frequency of grooming behaviour and low-pitched bleat displayed by the ewes towards their lambs as was found in Blackface ewes (Dwyer et al., 1999; Dwyer et al., 2004). Dwyer (2014) also suggested that the higher circulating oestradiol in Scottish Blackface ewes during late pregnancy may facilitate an increase in oxytocin receptors (OTR) in the limbic and hypothalamic areas of the brain that could result in an increased expression of maternal behaviour.

1.3.2 Maternal experience

Maternal inexperience also plays an important role contributing to the impairment of maternal behaviour. Primiparous females of many species including sheep are not as competent in displaying maternal care towards their offspring as multiparous mothers. Primiparous ewes, which are ewes giving birth for the first time, have been shown to groom or lick and produce low-pitched bleating as much as multiparous ewes (Dwyer & Lawrence, 2000). However, they are slower to begin licking and grooming their lambs after birth and make more high-pitched bleats than their multiparous counterparts (Dwyer & Smith, 2008). Primiparous ewes also display higher avoidance when lambs reach the udder in an attempt to suck (Alexander et al., 1993; Dwyer & Lawrence, 1998). The impairment of maternal care may result in a delay in sucking success by their lambs (Dwyer, 2003) and higher mortality in their offspring (Dwyer & Lawrence, 2005; Maria & Ascaso, 1999). In a recognition test with their own and alien lambs, it has also been demonstrated that primiparous ewes are slower to show preference for their own lamb and spent less time with the lamb 12 hours post partum compared to multiparous ewes (Keller et al., 2003). However, previous studies with other species have demonstrated an opposite effect where primiparous animals spent more time attending to their young compared to multiparous animals such as reported in pigs (Wischner et al., 2010), dogs (Guardini et al., 2015) and hamsters (Swanson & Campbell, 1979).

1.3.3 Temperament

The term temperament is often used to describe the individual variation in the expression of behaviour when responding or coping to various environmental challenges (Beausoleil et al., 2012; Boissy, 1995; Réale et al., 2007). Among the individual characters or traits commonly measured to assess the temperament of animals are fearfulness, activity, sociality and aggression (Boissy & Bouissou, 1995; Réale et al., 2007; Sinn & Moltschaniwskyj, 2005). From the evolutionary point of view, individual variation has long been an interesting subject although there are generally few relevant empirical studies that have been conducted on the topic as individual differences are often considered as non-adaptive variation or noise around the average mean (Dall et al., 2004).

The few studies which have been conducted previously have also reported an association between temperament and maternal behaviour in ewes. Ewes showing higher movement and vocalisation when being enclosed completely in box test (highly reactive) spent less time licking their lambs within the first hour after birth (Murphy et al., 1994), although this effect could not be replicated in a later study (Bickell et al., 2011). In addition, a more recent study showed a mild effect of temperament in an opposite manner where ewes which have been selected for highest social reactivity and plasma cortisol response during isolation (high-responsive ewes) spent more time grooming their lambs at parturition and vocalized more at 48 hour post-partum when separated from their lambs compared to low responsive ewes (Coulon et al., 2014). The difference found on the time spent licking or grooming the lambs may be due to the selection of ewes for the temperament trait. Although both Murphy et al. (1994) and Coulon et al. (2014) used isolation test which produced more or less similar outcomes, the latter study also took into account the plasma cortisol response in the ewes in categorizing the temperament of the ewes. It can be suggested that selecting for only one trait of temperament may not be adequate to determine the effect of individual variation on maternal behaviour as two traits may be associated at phenotypic level that are genetically or developmentally related to each other (Réale et al., 2007; Si et al., 2004)

1.3.4 Undernutrition during pregnancy

Maternal undernutrition has been associated with negatively altered maternal behaviour in sheep. Ewes offered a low intake (75% requirements) throughout gestation compared to ewes fed to requirements displayed less time licking their lambs after birth and were more aggressive towards the lamb (Dwyer et al., 2003). Ewes with twin lambs which had been subjected to a low level of nutrition six weeks prior to parturition displayed a higher proportion of deserting their lambs compared with ewes provided with a high level of nutrition (Putu et al., 1988). Ewes allocated to rations providing only 60% of individual metabolize energy (ME) requirement from day 1 to 90 of gestation displayed a higher frequency of withdrawal from their lambs in the 30 minutes after parturition than ewes provided with higher ration of ME (Muñoz et al., 2009).

Lower production of colostrum and milk has also been observed in undernourished ewes during pregnancy (Banchero et al., 2006; Bizelis et al., 2000). As starvation and hypothermia are important factors contributing to lamb mortality within the first seven days of life, early ingestion of sufficient colostrum and milk is crucial as it acts as fuel in maintaining the body temperature of the lamb (Charismiadou et al., 2000; Darwish & Ashmawy, 2011).

1.3.5 Difficult parturition

Difficulty during parturition or dystocia has also been reported to affect the expression of maternal behaviour. Ewes with difficult and prolonged parturitions are slower to start grooming their lambs, spend less time grooming, make fewer low-pitched vocalisations and have an increased occurrence of lamb rejection compared to ewes with short and uncomplicated parturitions (Dwyer, 2003; Darwish and Ashmawy, 2011). It is likely that the disturbed maternal behaviour displayed by the ewes is due to the stress they experienced during the prolonged or difficult birth where the cortisol produced during stress may somehow interfere with the release of oxytocin which is involved in parturition. There is also the possibility of the ewes becoming exhausted after a long labour and therefore can be slow to get up and groom the lamb. This might reduce the amount of bonding behaviour and may also increase the chance of rejection if the ewe does not interact with the lamb within the time window that she has available to form a bond.

1.4 Stress

Stress can be defined as the inability of an animal to cope with its environment which may affect the accomplishment of genetic potential such as growth rate, reproduction or milk yield (Dobson & Smith, 2000). It is a part of life and does not always produce damaging effects to an individual. However, when the stress response threatens the animal’s well-being and cause a deleterious effect, it is then defined as distress and this may compromise the welfare of the animal (Moberg, 2000).

Upon the perception of stress, the sympathetic nervous system (SNS) immediately releases norepinephrine from axon terminals while the adrenal medulla secretes epinephrine (Nelson, 2005; Tsigos & Chrousos, 2002). At the same time, the hypothalamic-pituitary-adrenal axis (HPA axis) is also activated releasing corticotropin-releasing hormone (CRH) from hypothalamus which will then trigger the anterior pituitary to release adrenocorticotropic hormone (ACTH) followed by the release of cortisol from the adrenal cortex within minutes of the onset of a stressor (Jankord & Herman, 2008). This immediate stress response was termed fight or flight response as it activates a series of physiological changes such as increasing in heart rate, blood flow and respiration rate to help animals survive by displaying those behavioural responses (Romero & Butler, 2007).

Stress can be either acute or chronic. Acute stress involves only a short-term exposure to a negative situation where the stressful event may happen for a short period or the animal is able to remove itself from the situation which results in quick and complete recovery (Dwyer & Bornett, 2004; Trevisi & Bertoni, 2009). However, chronic stress can be caused by few factors such as prolonged continuous exposure to the stressful condition, the presence of repeated acute stressors or longer term consequences of a short term but severe stressor present (Dwyer & Bornett, 2004; Trevisi & Bertoni, 2009). Chronic stress is one of the major challenges faced by farmed animals as the constraint of housing as well as the management and husbandry system may make it impossible for the animals to escape from the situation which therefore may result in the inability of the animal to perform natural behaviour (Wiepkema & Koolhaas, 1993).

As the SNS and HPA are activated during stress, many hormones such as catecholamines and glucocorticoids will rise temporarily in response to dealing with stressful situations (Möstl & Palme, 2002; Trevisi & Bertoni, 2009). The concentration of glucocorticoids in blood or saliva are widely used as an indicator of stress. However, the confinement or handling the animals during sample collection may be stressful for the animals and therefore may confound the results (Cook et al., 2000). As alternatives, non-invasive sampling procedures for glucocorticoid metabolites, such as in faecal samples, has gained increased importance in various biological fields e.g. wildlife studies and for welfare assessment (Möstl & Palme, 2002). Extra caution should be taken in interpreting stress from the concentration of glucocorticoids obtained as the hormones are also released in response to non-threatening arousal such as during exercise, courtship and copulation (Broom & Johnson, 1993). It is therefore important to observe other responses of stress such as behavioural responses as it may provide potential clues in determining the effects of putative stressors (Moberg, 2000).

1.4.1 Stress in sheep

Sheep may encounter various sources of stress on farm as a part of their social environment and normal husbandry routine, some of which may occur in combination (Figure 1.1). This includes exposure to heat and noise (Sevi et al., 2001a), repeated transportation and isolation (Price & Thos, 1980; Roussel et al., 2006), novel environment (Cockram et al., 2000), undernutrition (Dwyer et al., 2003), aversive handling by humans (Braastad, 1998; Hild et al., 2011; Mears et al., 1999), presence of dogs (Beausoleil et al., 2005) and overcrowding or frequent changing of social group by re-mixing (Jorgensen et al., 2009; Miranda-de la Lama et al., 2012; Sevi et al., 2001b).

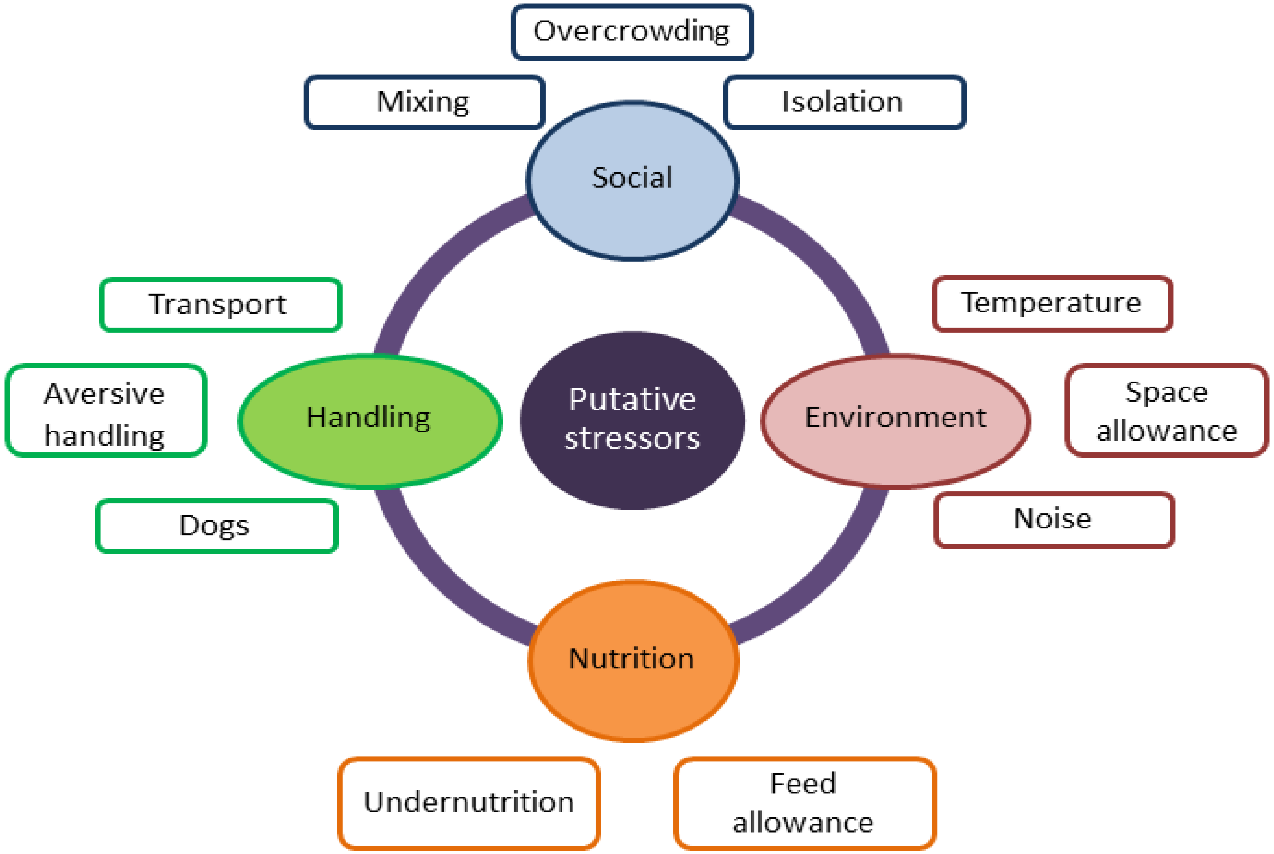

Figure 1.1. Putative stressors sheep may encounter on farm as part as their environment and daily management routine. The list is not exhaustive and the stressors may all occur at the same time or in different combinations.

Different types of stressful events may produce qualitatively different patterns of effects in both behaviour and physiology (Blanchard et al., 2001) which also depends on the coping ability and the early life experiences of an individual (Anisman & Matheson, 2005). For example, ewes subjected to regrouping and relocation had a higher number of aggressive interactions than ewes maintained in stable social groups (Sevi et al., 2001a), but also a higher number of sociopositive contacts (Averós et al., 2014). A similar result was obtained from a study of social mixing in lambs where, in addition to higher aggression, plasma cortisol levels were also increased (Miranda-de la Lama et al., 2012). Repeated transportation has been found to increase fear in ewes towards humans (Roussel et al., 2006), whereas poor ventilation in sheep housing has been shown to reduce the quality of ewe's milk and increase plasma cortisol levels (Sevi et al., 2001b; Sevi et al., 2003).

1.4.2 Stress during pregnancy in sheep

As mentioned above, the social environment of an animal may contribute to the stress it may experience on farm. There have been a considerable number of studies conducted on various farmed species which have shown negative impacts of prenatal stress on the offspring (Rutherford et al., 2012). In a review on cattle, stressors such as undernutrition, stocking density, stress from handling and transport as well as thermal stress when experienced by the pregnant cow have been shown to result in adverse consequences for the welfare of the offspring (Arnott et al., 2012; Rooke et al., 2015). However, the effect of environment prior to birth has not received as much consideration as postnatal events, although it has started to gain increased attention as of late (Rutherford et al., 2012).

It is common practice in the UK to house pregnant ewes indoor during winter, mainly from mid-gestation, to protect the ewes and their lambs from adverse conditions which may increased the mortality of the lambs (Winter & Fitzpatrick, 2007). In a survey conducted in the UK to investigate the hazards ewes may encounter on farm during pregnancy, 33% of farms were reported to house the pregnant ewes indoors prior to lambing while 43% of farms mixed the ewes into new social groups at least once during housing (DEFRA AW0509, 2013). Even with the high number of indoor housing of pregnant ewes, the effect of housing systems and the degree to which the maternal management could contribute to the stress response of the pregnant mother and the consequent effects on the lambs are still scarcely researched.

In a study investigating the effect of space allowance on pregnant ewes, ewes subjected to the smallest space allowance (1 m^2) only showed reduced movement and spent more time at the feeder but interestingly displayed higher positive social interactions (Averós et al., 2014). However, the lambs from these groups which were separated from their mother 24 hours after birth displayed higher frequency of immobility and vocalizations during social motivation and novel arena test which implies to be more fearful compared to lambs which remained with their mother (Averós et al., 2015).

In another study conducted by Roussel et al. (2004), pregnant ewes which were divided into high and low reactive temperament group based on their plasma cortisol level were exposed to repeated isolation with or without the presence of a dog. No effect of temperament was observed after exposure to repeated stressors and the ewes only reacted strongly to the first exposure to the stressor and completely habituated to the isolation stress with time. The lambs of these ewes, however, were heavier at birth, had a higher concentration of cortisol at 25 days of age and displayed higher exploration and locomotion activity at 8 months of age compared to control ewes.

As can be seen in these two studies, although only mild effects of maternal stress were observed in the ewes, the consequences of prenatal stress on the lambs were quite pronounced. A better understanding of the risk of poor housing management system towards pregnant ewes may lead to improvements that benefit the UK and worldwide livestock industry.

1.5 Gestational stress and maternal behaviour

Maternal behaviour can be affected by stress experienced by animals during pregnancy (Braastad, 1998; Nowak et al., 2000; Roussel et al., 2006, 2004). In both humans and animals, stress experienced during pregnancy generally results in physiological changes and disruptions in emotional function and cognitive ability in offspring; however, the effects of such stress in mothers are much less recognised (Baker et al., 2008).

A study on rats subjected to physical restraint during gestation showed that stressed mothers spent less time licking their pups (Baker et al., 2008). Rats exposed to overcrowding during the final week of gestation also had a weaker mother-pups interaction compared to control dams (Moore & Power, 1986). A higher offspring-directed-aggression was observed in guinea pigs exposed to repeated stressor during pregnancy than controls (Klaus et al., 2013). This study also reported a tendency of lower milk supply and longer pup suckling duration in stressed females. In a different study, besides reduced maternal behaviour, rats exposed to stress in late gestation also had a reduction in the density of oxytocin receptor (OTR) in the brain areas associated with the expression of maternal behaviour (Champagne & Meaney, 2006).

However, studies investigating the effect of pregnancy stress on maternal behaviour in sheep are still infrequent. The few studies within this area have produced inconsistent results on how stress may affect maternal care. For example, in a study looking at the effect of yarding and shearing in mid pregnancy, there was no significance difference in maternal behaviour scores between treatment groups (control, yarded and shorn ewes) (Corner et al., 2010). In another study however, ewes that had been aversively handled in pregnancy groomed their offspring for a longer duration than the ewes subjected to gentle human treatment (Hild et al., 2011). Ewes which had been exposed to various stressors such as social isolation, mixing and transport during pregnancy did not differ from control ewes in maternal behaviour displayed during the first 30 minutes after parturition and during selectivity test at 2 hours postpartum (Coulon et al., 2014). Since the current picture on the effect of stress on maternal behaviour in sheep is still unclear, more studies should be conducted to investigate the effect of pregnancy stress on maternal behaviour especially under conditions with normal husbandry of housed sheep. The mechanism of the neuroendocrine systems in the sheep regarding stress experienced during pregnancy is also inconclusive and need to be investigated further (Dwyer, 2014).

1.6 Conclusions

Lamb survival and growth is highly dependent on the maternal care provided by its dam. However, it has been shown in studies conducted in rodents that stress experienced during pregnancy may negatively impair maternal behaviour. As substantial number of pregnant ewes in the UK are housed prior to lambing, it is important to ensure that their physical and social environment during housing does not cause stress. Minimising stress to pregnant ewes will have welfare benefits not only to the ewes themselves, but potentially will improve lamb survival through improved maternal care. This will also, in turn, benefit the farmers in terms of farm and animal management as well as the revenue of the farm.

1.7 Overall objectives

The main aim of this project was to develop a scientific understanding of the impact of different indoor management systems during mid- to late- pregnancy on the expression of maternal behaviour in sheep. The response of pregnant ewes to their environment prior to lambing was also investigated to understand the magnitude of welfare challenge that the housing management system had on the ewes. This project was divided into two main studies. Both studies were designed to replicate the normal system practices on farm in the UK.

The aim of the first study was to investigate the effect of different space and feedface allowance as well as repeated mixing on pregnant ewes by behaviour and physiological measures to identify the mechanism and function of HPA axis in response to social stress. The housing conditions were chosen to represent the most common practices on UK farms following data collected in a farm practice survey (DEFRA Project AW0509). After parturition, the differences in maternal behaviour displayed by the ewes towards their lamb immediately from birth until lactation and the concentration of Immunoglobulin G (IgG) antibodies in the ewes’ colostrum as a result of maternal housing environment were also investigated.

For the second study, the aim was to investigate the behavioural and physiological response of pregnant ewes to an alternative system which was designed to eliminate competitive feeding behaviour during pregnancy and a negative system where the ewes were exposed to various putative stressors such as delayed feeding and repeated exposure to dogs, which may also occur on commercial farms. Similar to the first study, the effects of these different management systems on the maternal behaviour, concentration of IgG and additional physiological parameters were also investigated.

1.8 Hypotheses

For the first study, it was hypothesised that:

1. Ewes subjected to smaller space allowance and social mixing during pregnancy will show behaviour and physiological alterations, e.g. higher aggression and higher level of faecal cortisol metabolite suggesting that their welfare had been impaired
2. Ewes that have been exposed to reduced space during pregnancy will show less maternal care towards their lambs at parturition and lactation and will have a lower concentration of IgG in colostrum compared to ewes housed in more spacious conditions

As for the second study, it was hypothesised that:

1. Pregnant ewes allocated to the negative system will display altered behaviour, physiology and haematological parameters compared to control ewes whereas ewes allocated to the alternative system will show less responses indicative of stress.
2. Impaired maternal behaviour and lower concentration of IgG in colostrum as well as high concentration of glucocorticoid metabolite will be observed in negative ewes after parturition while no impairment will be observed in alternative ewes in all parameters recorded.

2. Effect of Different Social Environments on the Behaviour and Physiology of Pregnant Ewes

2.1 Introduction

In the UK, flocks of sheep are often kept indoors for at least part of the production cycle, mainly during winter from mid gestation until lambing in order to reduce the risk of lambs dying from exposure to adverse conditions (Winter & Fitzpatrick, 2007). However, having to relocate from extensive pasture into an indoor shed requires the ewes to adapt to the new environment. Apart from the novel environment, indoor housing imposes various types of challenges such as mixing with unfamiliar conspecifics at close proximity, limited availability of floor and feed space allowances, changes to the diet, as well as increases in human contact and handling.

In farm animals, stress experienced during gestation has been known to induce considerable impacts on the physiology and behaviour of the offspring. Studies in pigs report that gilts mixed with older sows during pregnancy were shown to produce less active and less vocal piglets during weaning (Ison et al., 2010) along with an increase in the tendency for anxiety-related behaviour in female offspring (Rutherford et al., 2014). The effects of inadequate housing environment during pregnancy in sheep have also been investigated. For example, lambs born to ewes kept at reduced space allowance per ewe during gestation and separated from their mother after birth, showed more fearful behaviour during novel arena and social motivation tests compared to lambs whose mothers were kept in larger space allowance (Averós et al., 2015). Ewes which were aversively handled during pregnancy were also shown to produce lambs with increased fearfulness (Coulon et al., 2011).

However, the studies on the impact of housing environment during pregnancy on the ewes themselves during both gestation and lambing are still insufficient. From the small number of studies that have been conducted on pregnant ewes, space limitations including smaller floor area and feed trough per ewe have been associated with altered resting patterns (Averós et al., 2014) as well as increase in displacement during feeding (Bøe & Andersen, 2010). Ewes that have been aversively handled in late pregnancy were also recorded to show a tendency to have increased concentration of plasma cortisol (Hild et al., 2011). In addition, maternal experience also had an effect on the behavioural responses to possible stressors. Inexperienced ewes (not given birth before) were significantly more fearful in a surprise effect test as well as to the presence of a human compared to multiparous (given birth before) ewes (Viérin & Bouissou, 2002).

Progesterone and oestradiol play a variety of pivotal roles in ensuring a healthy pregnancy for the mother. During the first half of pregnancy, the concentration of plasma progesterone in ewes will increase gradually until day 85-90 post conception where it will start to increase steadily and peak at day 125-130 (Bassett et al., 1969; Thorburn, Challis, & Currie, 1977) of gestation before declining in the last few days before parturition (Bassett et al., 1969). As for oestradiol, the concentration remains at low levels until around mid-pregnancy (week 10-12 of gestation) where it will gradually increase before it undergoes rapid rise during the final days of gestation (Dwyer et al., 2004; Dwyer & Smith, 2008). The concentration of oestradiol during the final days of gestation is associated with the display of maternal behaviour by the ewes towards their lambs after parturition (Dwyer et al., 2004). Suffolk ewes, which do not show as competent maternal behaviour as Scottish Blackface ewes, have significantly lower concentrations of circulating oestradiol than Scottish Blackface ewes (Dwyer et al., 2004; Dwyer & Smith, 2008). Ewes subjected to undernutrition during pregnancy had significantly lower oestradiol:progesterone value and negatively altered maternal behaviour after birth compared to well-fed ewes (Dwyer et al., 2003). However, the effect of social environment in indoor housing of pregnant ewes on the concentration of oestradiol and progesterone, which may or may not affect maternal behaviour, has not been researched so far.

Though inadequacy in indoor housing conditions may have negative consequences for animal welfare, the degree of severity experienced by ewes during gestation and after lambing under commercial housing conditions is still unclear. The first aim of this chapter was to investigate whether exposure to different housing conditions from weeks 11 to 18 of gestation has an impact on the behaviour and physiology of the pregnant ewes. In addition, a second aim was to determine if the behaviour and physiology of pregnant ewes under these conditions differ according to parity as primiparous ewes had not experienced housing conditions before. The parameters presented in this chapter include weight and body condition score, aggressive behaviour at feedface, general pen behaviour, the concentration of faecal glucocorticoid metabolite, and plasma oestradiol and progesterone.

2.2 Materials and methods

This study was conducted from early February until end of March 2014, at Woodhouselee Farm near Edinburgh. All procedures in this study were approved by the Scotland's Rural College (SRUC) Animal Experiments and Ethics Committee (approval ID: ED AE 50-2013) and were performed under UK Home Office license, following the regulations of the Animals (Scientific Procedures) Act 1986.

2.2.1 Animals, facilities, and management

Scottish Mule ewes (Scottish Blackface X Blue-faced Leicester) in their first (primiparous) and second pregnancy (multiparous) were used in this study. All the ewes came from SRUC's own Woodhouslee flock. The ewes were naturally mated to 1 of 2 Suffolk rams in November 2014. For the mating process, the ewes were initially put into small groups of 30-40 ewes. A ram was then placed together with the ewes for approximately four to five weeks. The ram was fitted with a marking harness which contained a coloured crayon resulting in a coloured rump on ewes that have been mounted. The colour of the crayon was changed every 10 days to facilitate the estimation of the date of parturition.

At approximately 10 weeks of gestation, all the ewes from Woodhouslee flock underwent transabdominal ultrasonography for pregnancy determination and to identify the number of lambs they carried. The ultrasonography was carried out by experienced contractor hired by SRUC. In the scanning procedure, twin-bearing ewes were identified and 77 ewes were then chosen to be used in the experiment which comprised of primiparous (n = 41) or multiparous (n = 36).

Ewes were brought indoors in week 10 of gestation following ultrasonography, when they were weighed and their body condition was scored. A weigh crate was placed in front of the pen and the ewes were ushered into the crate one ewe at a time to measure body weight. Body condition of the ewes was also scored while they were inside the crate. The condition scoring (measurement of relative fat and muscle over the lumbar vertebrae on a scale from 0 (emaciated) to 5 (obese)) was performed by an experienced technician using manual palpation over the lumbar spine.

In order to identify the ewes individually, they were also marked with a unique number on their lateral sides using a marker spray designed for use on animals (Super Sprayline Stock Marker, Ritchey, New Zealand). They were then taken into the experimental shed, which was straw-bedded, and the ewes were provided with ad libitum access to hay and water throughout the experiment. Concentrate feeds (18% crude protein, Premium 18 Nuts, Harbro Ltd., Scotland) were provided starting week 14 of gestation (approximately 300g/ewe) which was given once per day in the morning. The amount of concentrate provided was doubled to 600g/ewe at week 16 which was given twice a day in the morning and afternoon (300 g/ewe per meal). At week 18, the concentrate feeds provided were then increased to 800g/ewe which were also given in the morning and afternoon. Ewes were vaccinated with Heptavac P Plus (Intervet, Ireland) at week 17 of gestation, which was administered by subcutaneous injection in the lateral side of the upper neck by an experienced technician to provide high maternally derived antibodies (MDA) to protect the lamb against clostridiosis and pasteurellosis.

Ewes were then allocated into experimental pens (described below in 2.2.2) as they entered the shed after selection. After the data collection for the assessment of impact of housing during pregnancy ended, ewes remained in the same pen until lambing.

2.2.2 Social stress treatment

The experiment and data collection began at week 11 of gestation until 18 week of gestation (length of gestation = 20 weeks). All the ewes were assigned to one of two treatment groups at week 10 of gestation, with multiparous and primiparous ewes were balanced within the treatment pens (Table 2.1) which differed in space allowance per ewe, length of feedface per ewe and occurrence of social mixing:

- **Control (C):** space allowance of 2.5 m2/ewe, feedface allowance of 71 cm/ewe, and stable social group (ewes remained within their allocated pen group until lambing), or

- **Restricted Space – Mix group (RS-Mix):** space allowance of 1.27 m2/ewe, feedface allowance of 36 cm/ewe, and subjected to two social mixing events during the experiment.

Table 2.1. Numbers of multiparous and primiparous ewes in each treatment group (Control group (C) and Restricted Space – Mix Group (RS-Mix)).

	Control	RS-Mix
Multiparous	20	16
Primiparous	22	19

There were a total of six control pens (2.3m X 7.55m) and five RS-Mix pens (2.3m X 3.85m) with seven ewes in each pen.

Ewes in RS-Mix groups were exposed to social mixing on the Monday of weeks 13 and 15 of gestation where new groups consisting of different individuals were established. As there were only 5 pens of RS-Mix group, each new group was composed of 1 original member, 1 pair of ewes from 2 different pens and another 2 ewes from the other 2 pens.

2.2.3 Weight and body condition score

Body condition score and body weight of the ewes were first measured before the ewes were assigned to treatment groups at week 10 of gestation. Both body weight and condition score were measured again on week 13, 15 and 17 of gestation, as well as at the end of week 18 of gestation, which was the final day of observation of the ewes before parturition.

2.2.4 Behavioural observation

2.2.4.1 Aggressive behaviour at feedface

Thirty minutes continuous observations on the frequency of behaviours and interactions (Table 2.2) at the feedface were recorded at 14, 16, and 18 weeks of gestation, beginning immediately after concentrate feed was placed in the feed troughs at the morning feed. Two pens were observed each day (1 Control & 1 RS-Mix group) except on Friday where three pens were observed. Observations were made using a camcorder (Canon Legria HFM52, Canon Inc., Japan) placed in front of the observed pen starting at 0800 every morning. Prior to recording, the camcorder was positioned appropriately in front of the pen to make sure that the whole feed trough and the feeding ewes were captured.

Table 2.2. Definition of ewe behaviours recorded at the feedface.

Behaviour	Definition
Join Feedface	When an animal physically moves in from the back of the pen to join or enter the feedface from approximately a ewe length away.
Push-In	An animal forces itself between 2 other animals (< ewe body width gap between animals) or between an animal & barn equipment (fence) at the feedface.
Penetrate	An animal forces itself between 2 other animals (no gap between animals) or between an animal & barn equipment (fence) at the feedface.

Failed penetrate	A ewe has an unsuccessful attempt to get in between 2 other ewes or between an animal & barn equipment (fence) at the feedface.
Displace	Physically forcing another sheep to leave her feeding place by butting, hitting, striking, thrusting, pushing or penetrating the receiver with forehead, horns, horn base or any other part of the body with a forceful movement resulting in the receiver giving up its position (walking away for at least half an animal-length or stepping aside for at least one animal-width).
Half Displace	When a push leads to a ewe moving or step back approximately half body length from the feedface, but not fully displaced or left the area.
Leave Feedface	Ewe voluntarily leaves the feedface without any interaction with another animal.
Push	Forcefully moving another ewe while at the feedface.
Butt	Contact with another ewe either head-to-head or short and forceful contact with the head towards other part of the receivers body.
Prod	One ewe uses her hoof to tap/prod/kick the back/side of another ewe .
Mount	Jumping on another sheep's back.
Back Press	One ewe pressing down on the backside of another ewe with its jaw/head.

2.2.4.2 General pen behaviour

Live scan sampling of pen behaviours (Table 2.3) on each individual ewe was conducted from week 12 to week 18 of gestation. On week 12 of gestation when the concentrate feed has not been given to the ewes, the live scan sampling started at 0850 on the observation days. From week 14 of gestation when the concentrate feed has been supplied to the pregnant ewes, the scan sampling started 15 minutes after the end of feedface observation on the same pens each day with the addition of two or three other pens (four or five pens were scanned each day) such that each pen was observed twice per week. A total of 8 scans at 10 minutes interval were conducted on each observation day beginning from 0850 and ending at 1010.

Table 2.3. Definition of ewe behaviour recorded using scan sampling during gestation

Level	Category	Definition
Posture	Standing	Ewe is standing on all four legs, body clear of the ground.
	Walk	Ewe in motion, moving from one location to another.
	Lying	Ewe's body is in contact with the ground.
	Sitting	Ewe has rear end in contact with ground, but supporting some weight on straight front legs.
	Kneeling	Ewe is kneeling on front legs, supporting some weight on back legs.
Behaviour	Idle	No activity, motionless, head up.
	Head down idle	No activity, motionless, head down (resting on substrate).
	Feed	Ewe in front of the feeder biting, chewing or pulling on hay.
	Feed Sub	Ewe is biting, chewing or pulling on substrate material, not normal allocated food.
	Drink	Ewe standing in front of the drinker and is seen to consume water or with its nose within 10 cm of from the drinker.
	Ruminate	Ewe making chewing movements with its mouth while lying or standing.
	Lick (self)	Ewe licking or scratching a part of the body with tongue or teeth.

Rub (against object)	Rubbing self against objects.
Groom (other)	Ewe licking or pulling feed from the wool of other animal.
Agonistic	Includes all forms of aggression towards another ewe (pushing; mounting, kicking, butting, threat, block; displacement).

2.2.5 Faecal collection, extraction and hormone assay

2.2.5.1 Collection of faecal samples

Faecal samples were collected per rectum between 0900 to 1100 hr on week 11, 13, 15 and 17 of gestation from 72 ewes across 11 pens (five ewes were not able to be sampled due to Home Office license constraints). Faecal samples were collected by inserting index and middle fingers (with a clean glove on) into the rectum of each ewe, one finger at a time and removing the available faecal matter with both fingers. Faecal material was also collected immediately from the ground when the ewes were seen to have naturally deposited their faeces while the technician/researcher was in the pen. Each sample was placed into a labelled plastic bag, homogenised by hand for ease of processing and then frozen at -20 °C until further analysis.

2.2.5.2 Faecal extraction

Prior to extraction, the homogenised faecal samples were brought to room temperature for 30 minutes. 0.5 g of the defrosted sample was transferred into a 15ml centrifuge tube before 5 ml of 80% methanol was added. Tubes were vortexed for 30 minutes and centrifuged at 2500 g for 15 minutes (Z200A, Hermle, Germany), before 1 ml of the supernatant was removed to a clean Eppendorf tube.

Faecal extracts were assayed for immunoreactive glucocorticoid metabolites using an EIA for 11-oxoetiocholanolon cortisol metabolite (Palme and Mostl, 1997;

Mostl et al, 2002). This method has been successfully validated for the evaluation of adrenal activity in sheep (Palme and Mostl, 1999).

2.2.5.3 Enzyme immuno-assays (EIA) for faecal glucocorticoid metabolites

The standard, antibody and enzyme label that were used in this study were supplied by Professor Rupert Palme from University of Veterinary Medicine, Vienna, Austria.

First, the 96 well microtitre plates (F96 MaxiSorp, Nunc, Denmark) were coated with 250µl/well of Protein A solution (cell surface receptor by *Staphylococcus aureus* derivative; P-7837, Sigma, Germany) at 2µg/ml in coating buffer (15mM $NaCO_3$, 34 mM $NaHCO_3$; pH 9.6) and incubated at room temperature overnight. The Protein A solution was discarded the next day and plates were then blocked with 300 µl/well blocking buffer (20mM Tris, 300mM NaCl, 1% BSA; pH7.5) for at least 3 hours at room temperature. The plates were washed three times with washing buffer (0.02% Tween20) before being loaded with samples. 50µl of each sample, diluted 1:10 in assay buffer (20mM Tris, 300mM NaCl, 0.1% BSA, 0.1% Tween80; pH 7.5) was pipetted into appropriate duplicate wells on the coated microtiter plates. The standard (11-oxoaetiocholanolone, 5β-androstane-3α-ol-11,17-dione) was prepared in seven concentrations (2.56-500 pg/ml in a 1:2.5 dilution series) and added to the appropriate wells in duplicate with a volume of 50µl/well. 100µl of enzyme label (11-oxoaetiocholanolone-3-glucosiduronate-DADOO-biotin; diluted 1:250,000 in assay buffer) were then dispensed into all wells followed by 100µl of antibody (rabbit antibody against 11-oxoaetiocholanolone-3-HS:BSA; diluted 1:20,000 in assay buffer). Assay buffer was also used as negative control whereas two dilutions of pool (1:10 & 1:20) made up of faecal samples from 12 different ewes were used as positive controls and placed at the front and back of each plate in duplicate. The plates were sealed and incubated overnight at 4^{o}C on a plate shaker. The plates were then washed four times with wash buffer before adding 250µl of diluted streptavidin conjugate solution (Streptavidin-POD conjugate, Roche 11089153001, 17 µl/ml in assay buffer) into each well and incubated at room temperature for 45 min on a plate shaker. After incubation, the plates were washed again and 250µl of Tetramethylbenzidine (TMB) substrate buffer (10mM CH3COONa, pH 5.0; 0.002% H2O2, 0.006% TMB) was dispensed into each well. The plates were sealed, covered with foil and incubated again for 45 minutes on a plate shaker at room temperature before adding 50µl of stop solution (10% H_2SO_4) into each well to stop the reaction. The absorbance was measured at 450 nm on a Multiskan FC spectrophotometer (Thermo Scientific, UK) using SkanIT Software 2.5.1. From the ELISA analysis, coefficient of variation (CV%) of intra-plates and inter-plates were shown to be 7.2% and 18.9% respectively.

2.2.6 Plasma oestradiol and progesterone

Blood samples collected in 4 ml vacutainers (BD Vacutainer®, Plymouth, UK) via jugular venepuncture were taken on the 13, 15, and 17 week of gestation and were always sampled right after their faecal samples were collected. After every two or three pens were sampled, the vacutainers were centrifuged at 2500 rpm for 20 minutes using a centrifuge machine (Mistral 3000i, MSE, UK) located in the experiment shed. The plasma obtained were then pipetted into Eppendorf 1.5ml tubes and frozen at -20 °C until further analysis.

Plasma oestradiol and progesterone were measured using commercially available ELISA kit (Cusabio, Wuhan, China). Both kits provided a 96 wells microtiter plate which has been pre-coated with goat-anti-rabbit antibody. A blank well without any solution was set on each plate. 50 µl of samples (diluted 1:2 for oestradiol assay and 1:10 for progesterone assay in phosphate buffered saline (PBS)), 50 µl of six standards (different concentrations, provided with the kit) and a positive control (350 pg/ml for oestradiol assay and 3.5 ng/ml for progesterone assay) were filled into the appropriate wells on the plate. Pools that were prepared using samples from 16 ewes (diluted with the same ratios for each oestradiol and progesterone assay) were also placed in the wells at the beginning and end of each plate as a reference.

Except for blank, 50 µl of HRP-conjugate was then added to each well. This was followed by adding the same amount of antibody and the plate was incubated for one hour at 37°C. After a total of three washes using wash buffer, 50 µl of both Substrate A and Substrate B were added to each well before the plate was incubated again in the dark for 15 minutes at 37°C. After 50 µl of stop solution has been added, the optical density was read at 450 nm on a Multiskan FC spectrophotometer (Thermo Scientific, UK) using SkanIT Software 2.5.1. From ELISA analysis conducted, coefficient of variation (CV%) of intra-plates and inter-plates were shown to be 25.4% and 47.09% respectively for plasma oestradiol, whereas coefficient of variation (CV%) of intra-plates and inter-plates for plasma progesterone were 21.4% and 38.87% respectively.

2.2.7 Statistical analysis

From the original sample of 77 ewes, only data from 71 ewes were analysed. Six ewes (1 from RS-Mix & 5 from Control group) were excluded from the analysis as one aborted at week 19 of gestation, one died due to prolapse in the middle of gestation, one subsequently gave birth to triplets instead of twins, and another three ewes were excluded due to ill thrift throughout the experiment.

Daily body weight change was calculated as the difference between two points of weighing divided by the number of interval days. For scan sampling data, to obtain weekly measures of ewe behaviour, the percentages of occurrence of behaviours were calculated individually per ewe for the 80 min observation conducted each day (8 scans). The weekly values were obtained from the two observations per pen per week.

Due to the low frequency of each different type of ‘aggressive behaviour’ during the 30 min observation when concentrates were given, the data for push, butt, prod, mount, backpress, push-in, penetrate, displace and half displace behaviour were combined to make up the total of aggressive behaviour occurring at the feedface. The proportion of ‘free join’ (from the total of all types of join to the feedface: join, push-in and penetrate) and ‘free leave’ (from the total of all types of leaving pen: leave voluntarily and being displaced) were also calculated and used in analysis. However, for general pen behaviour recorded by scan sampling, feeding, ruminating, idle, standing and lying were the only behaviours analysed since all other behaviours recorded had a very low frequency.

Data were checked for normality and transformed as necessary. For all transformed data, the mean are reported together with Confidence Interval (CI) instead of using Standard Error of Mean (SEM) as in untransformed data. Body weight and condition score and physiological data (concentrations of faecal glucocorticoid metabolites, plasma oestradiol and progesterone) were analysed by linear mixed models using the Restricted Maximum Likelihood (REML) procedure. Data for plasma oestradiol were log (base 10) transformed whereas data for plasma progesterone were square root transformed prior to analysis. Aggressive behaviour at feedface was analysed using a Generalized Linear Mixed Model (GLMM), fitting a Poisson distribution with a Logarithm function. The proportion of free join and free leaves which occurred at the feedface were also analysed using a GLMM, fitting a binomial distribution with a Logit function. General pen behaviour by scan sampling was also analysed using GLMM but fitting a binomial distribution with logit function. Gestation week, parity and treatment as well as the interaction between them were fitted as the fixed effects whereas pen and individual ewe were fitted as random effect to account for repeated measures during data collection over the gestation period. Where differences were found, post-hoc comparisons were made using Fishers' Least Square Differences (LSD) tests. All analyses were conducted in GenStat (16th edition) software.

2.3 Results

2.3.1 Weight and body condition score

Throughout the experiment, there was no significant difference in weight change by treatment group (mean weight (kg) change (SEM): Control: 0.24 kg (0.012), RS-Mix: 0.25 kg (0.012); $F_{1,12.5}$ = 0.12, P < 0.731). On the other hand, Multiparous ewes were significantly heavier than Primiparous ewes when they were weighed at week 10 of gestation prior to being assigned to either one of the two treatment groups (mean weight (SEM): Multiparous: 77.98 kg (0.92), Primiparous: 72.52 kg (0.90); $F_{1,71.8}$ =

26.06, P < 0.001). Primiparous ewes were found to gain less weight in week 13 and 15 of gestation and some even lost weight in week 13 of gestation compared to multiparous ewes whose body weight increased in all weeks (Figure 2.1; $F_{3,224.7} = 3.58$, P = 0.015).

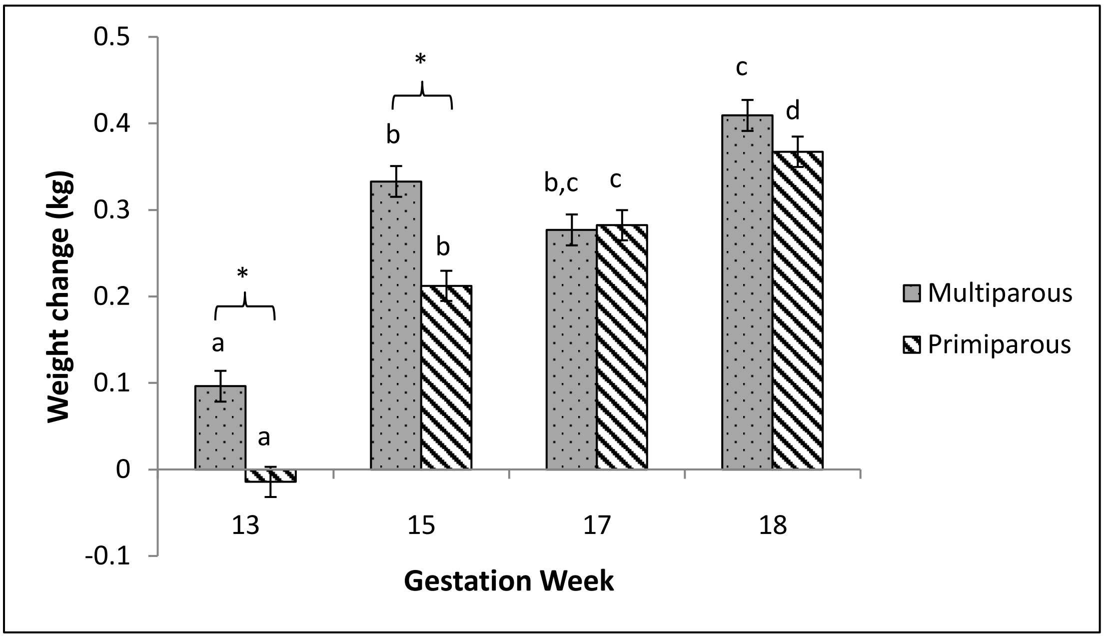

Figure 2.1. Change in daily weight (kg) with increasing gestational week for multiparous and primiparous ewes. Data presented are mean weight change with SEM as error bars. Bars with different letter superscripts and * indicate significant difference between gestation week and parity respectively at P < 0.05 level.

Body condition score was also not significantly affected by either treatment or parity (Table 2.4; average overall BCS = 3.2).

Table 2.4. Mean of Body Condition Score (BCS) in pregnant ewes based on treatment groups and parity.

	BCS (SEM)	P-value
Treatment		
Control	3.183 (0.052)	$F_{1,37.4} = 0.29$, P = 0.593
RS-Mix	3.142 (0.054)	

Parity		
Multiparous	3.142 (0.052)	$F_{1,68.2}$ = 0.35, P = 0.559
Primiparous	3.183 (0.049)	

2.3.2 Aggressive behaviour at feedface

Multiparous ewes displayed significantly higher aggression at the feedface (frequency/30 mins) over the treatment period than primiparous ewes (mean frequency (CI range): Multiparous: 2.69 (2.01-3.6), Primiparous: 1.77 (1.32-2.01); $F_{1,70.8}$ = 4.2, P = 0.044). Aggressive behaviour performed by ewes from RS-Mix group declined over the three observation periods compared to the Control group which was more constant over time (Figure 2.2.; $F_{2,145.4}$ = 3.17, P = 0.045). Post-hoc test also revealed that significantly higher aggressive behaviour was displayed by RS-Mix ewes compared to control ewes at 14 week of gestation (Figure 2.2).

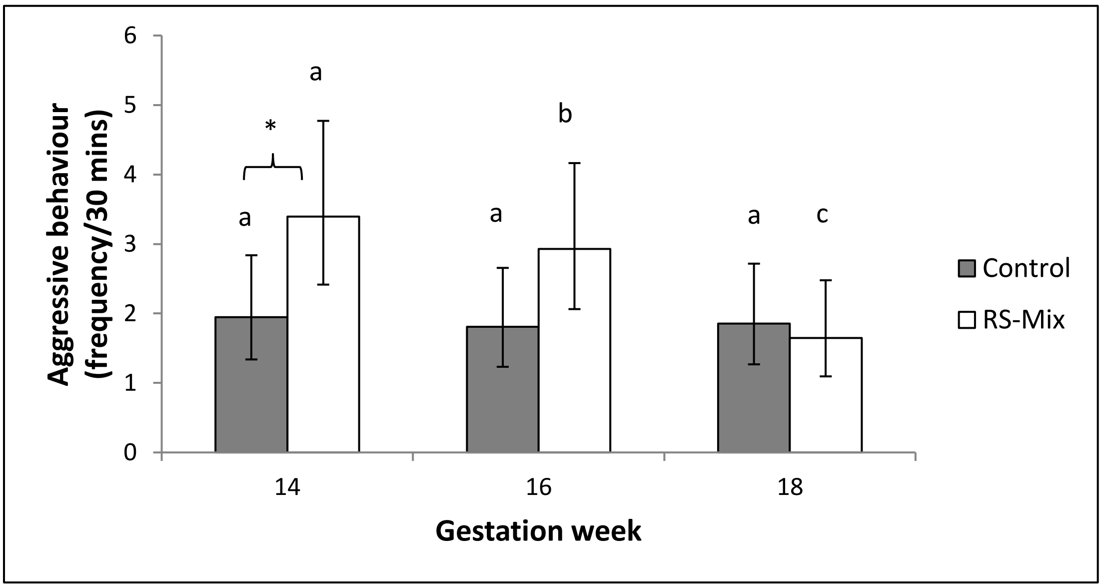

Figure 2.2. Influence of treatment groups on the frequency of aggressive behaviour displayed by pregnant ewes in 30 minutes at the feedface at weeks during gestation. Data presented are means (±CI). Bars with different letter

superscripts and * indicate significance different between gestation week and treatment respectively at P < 0.05 level.

Overall, ewes from RS-Mix group showed a significantly lower proportion of free join to the feedface (out of all types of join: join, push-in and penetrate) compared to Control ewes (mean proportion (CI range): Control: 0.91 (0.88-0.93), RS-Mix: 0.76 (0.71-0.81); $F_{1,11.7}$ = 30.4, P < 0.001). Multiparous ewes also displayed a significantly lower proportion of free join compared to Primiparous ewes (mean proportion (CI range): Multiparous: 0.81 (0.77-0.85), Primiparous: 0.88 (0.84-0.91); $F_{1,59.6}$ = 5.5, P = 0.022). However, no effect of treatment and parity were found on the proportion of free leave by the ewes (Table 2.5).

Table 2.5. Mean proportion of Free Leave at feedface in pregnant ewes based on treatment groups and parity.

	BCS (SEM)	P-value
Treatment		
Control	2.911 (0.30)	$F_{1,8.4}$ = 1.74, P = 0.222
RS-Mix	2.326 (0.32)	
Parity		
Multiparous	2.772 (0.26)	$F_{1,55.6}$ = 1.75, P = 0.191
Primiparous	2.464 (0.24)	

2.3.3 General pen behaviour

There was no treatment effect observed on feeding behaviour (Percentage (CI): Control: 30.2% (25.6-35.2), RS-Mix: 26.5% (21.9-31.6); $F_{1,9.6}$ = 1.11, P = 0.318). However, multiparous ewes were observed to feed more frequently compared to primiparous ewes throughout the experiment (Figure 2.3; percentage (CI):

Multiparous: 30.6% (26.6-35), Primiparous: 26.1% (22.5-30); $F_{1,71.7}$ = 5.56, P = 0.021).

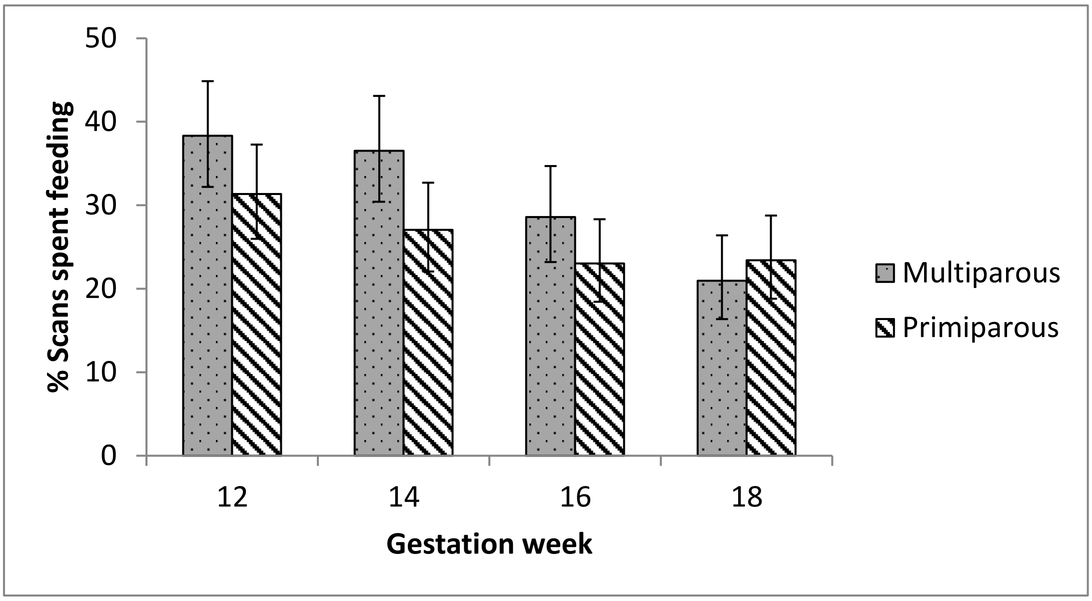

Figure 2.3. Effect of parity on percentage of observations where ewes were feeding during observation at different gestation weeks. Data presented are mean percentage (±CI). Overall multiparous ewes fed significantly more than primiparous ewes (P<0.021).

For ruminating behaviour, there was a significant interaction between treatment and gestation week with ewes from both treatments significantly displayed higher ruminating behaviour at week 12 of gestation before declining to week 16 (Figure 2.4; $F_{3,290.1}$ = 7.26, P < 0.001). However at week 18 of gestation, ewes from RS-Mix group displayed significantly higher frequency of ruminating than Control ewes (Figure 2.4).

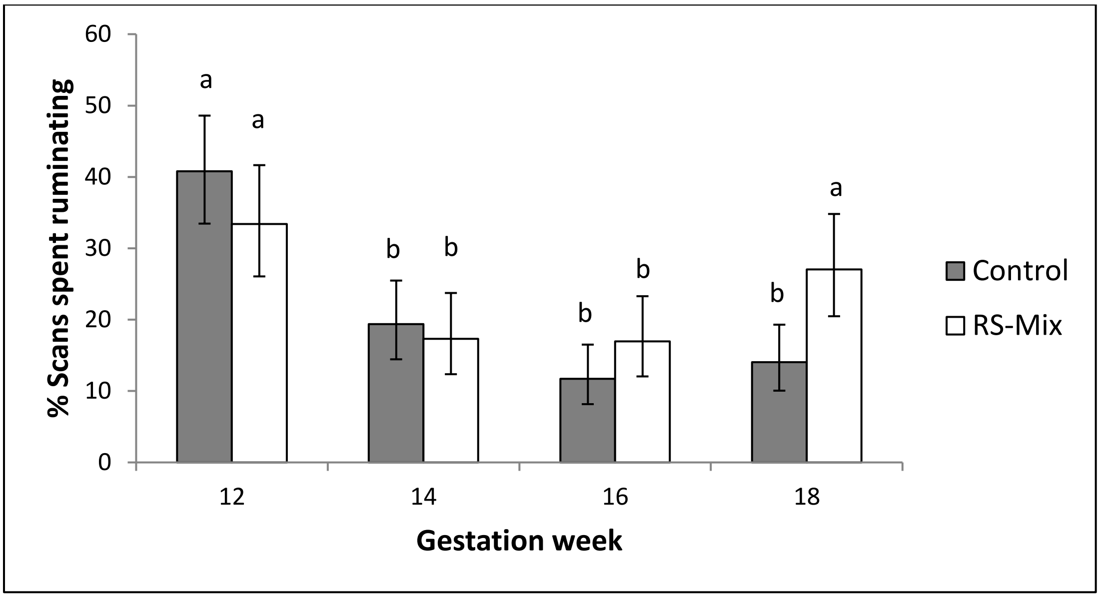

Figure 2.4. The effect of treatment group on percentage of observation where ewes were ruminating at different gestation week. Data presented are mean percentage (±CI). Bars with different letter superscripts indicate significance treatment within treatment group and between gestation week at P<0.005.

Primiparous ewes also spent significantly more time ruminating compared to multiparous ewes over the experimental period (Figure 2.5; $F_{1,299.0}$ = 9.54, P = 0.002).

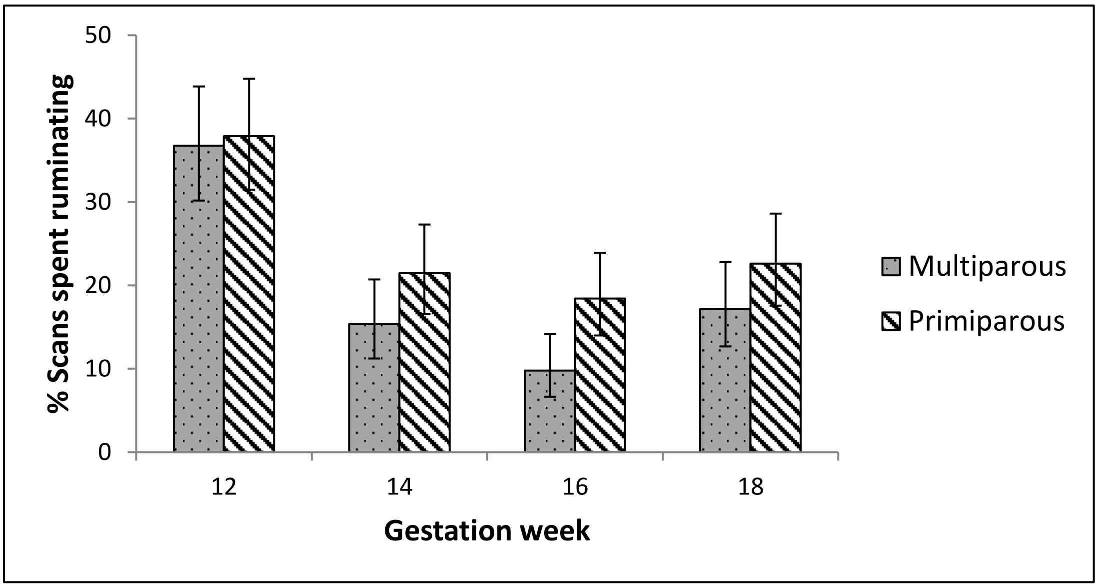

Figure 2.5. Effect of parity on the percentage of observation that ewes were ruminating at different gestation week. Data presented are means percentage (±CI). Overall primiparous ewes ruminated significantly more than multiparous ewes (P<0.002).

Control ewes spent less time idle at 12 week of gestation than in other weeks and idling time linearly increased before remaining constant at the end of the observation period (Figure 2.6; $F_{3,219.5}$ = 4.59, P = 0.004). RS-Mix ewes also spent less time idle at 12 week of gestation, but increased at week 14 of gestation and remained constant throughout observation (Figure 2.6). Primiparous ewes were observed to idle more frequently compared to Multiparous ewes at week 12 of but did not differ thereafter (Figure 2.7); $F_{3,223.7}$ = 4.58, P = 0.004).

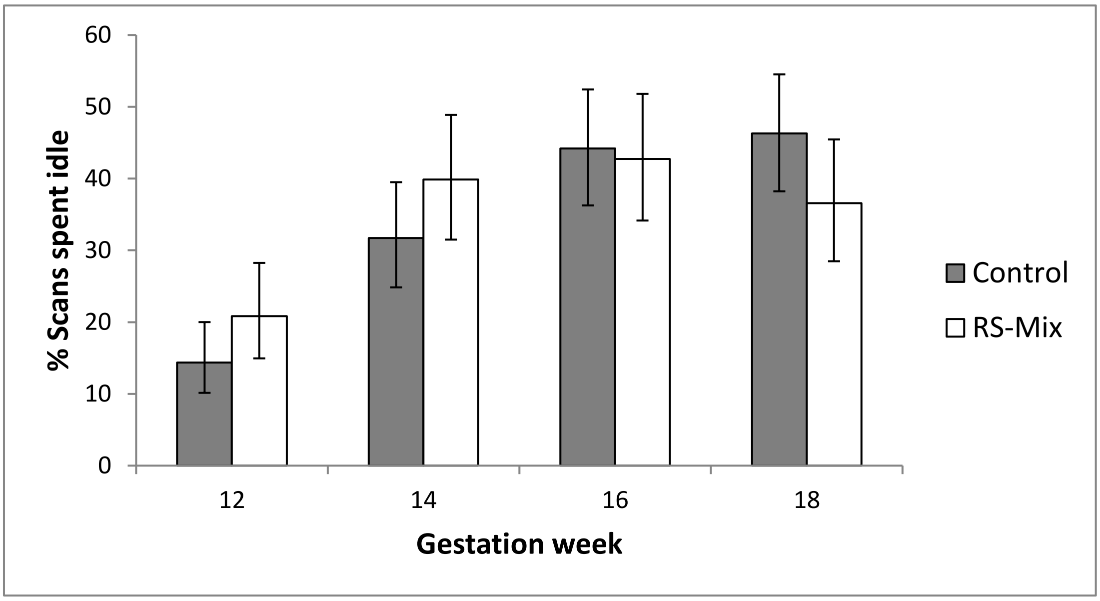

Figure 2.6. Mean percentage (±CI) of observation spent idle by Control and RS-Mix ewes with increasing gestation week. Both Control and RS-Mix ewes spent the least time idling at week 12 of gestation before increasing at week 14 of gestation which remained constant until the end of experiment (P = 0.004).

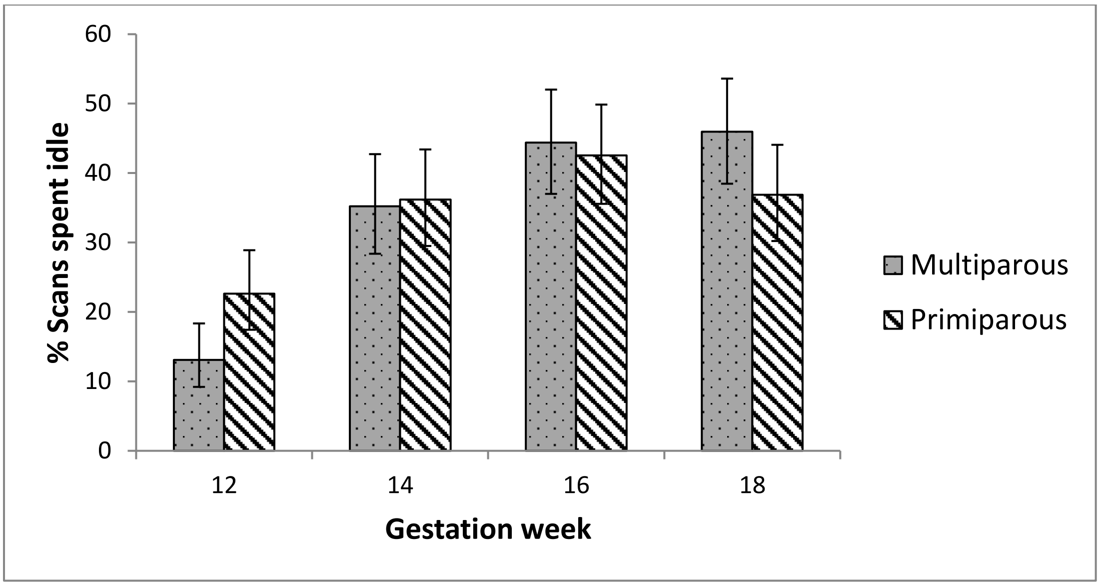

Figure 2.7. Mean percentage (±CI) of observation where multiparous and primiparous ewes spent time idle with increasing gestation week. Primiparous ewes displayed significantly higher idling behaviour at week 12 of gestation compared to multiparous ewes (P=0.004).

There were no significant differences by treatment or parity in the frequency with which ewes were observed standing and lying (Table 2.6).

Table 2.6. Mean percentage of standing and lying (with CI) observed in pregnant ewes during scan sampling based on treatment groups and parity.

	Standing	Lying
Treatment		
Control	45.4% (39.9-51.1)	45.7% (41.2-50.2)
RS-Mix	42.1% (36.2-48.2)	41.7% (36.9-46.6)
	$F_{1,12.6}$ = 0.77, P = 0.396	$F_{1,11.1}$ = 1.39, P = 0.263
Parity		
Multiparous	46.2 (40.8-51.6)	43.9% (39.5-48.5)
Primiparous	41.4 (36.4-46.5)	43.4% (39.2-47.7)
	$F_{1,71.3}$ = 2.12, P = 0.15	$F_{1,72.5}$ = 0.04, P = 0.837

However, further into gestation, ewes showed less frequency of standing and were seen to lie more frequently (Figure 2.8; $F_{3,218.6}$ = 9.38, P < 0.001).

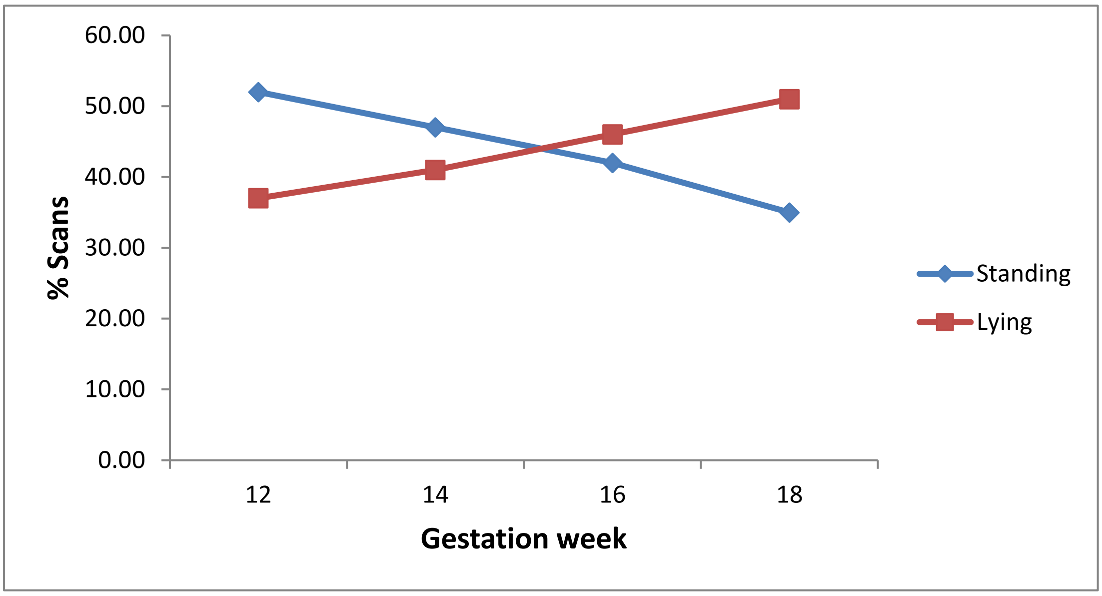

Figure 2.8. Mean percentage (±CI) of observation where ewes were observed to lie more and stand less with increasing gestation week (P<0.001).

2.3.4 Faecal glucocorticoid metabolites (FGM)

There was no effect of treatment on the concentration of FGM throughout gestation (Concentration (ng/ml) (SEM): Control: 53.14 (3.88), RS-Mix: 57.22 (3.86); $F_{1,21.9}$ = 0.52, P = 0.48). However, there was a significant interaction between gestation stage and parity in the concentration of faecal glucocorticoid metabolites (FGM) with primiparous ewes having higher FGM concentration in week 11, 13 and 15 of gestation compared to multiparous ewes (Figure 2.9; $F_{3,178.7}$ = 3.64, P = 0.014).

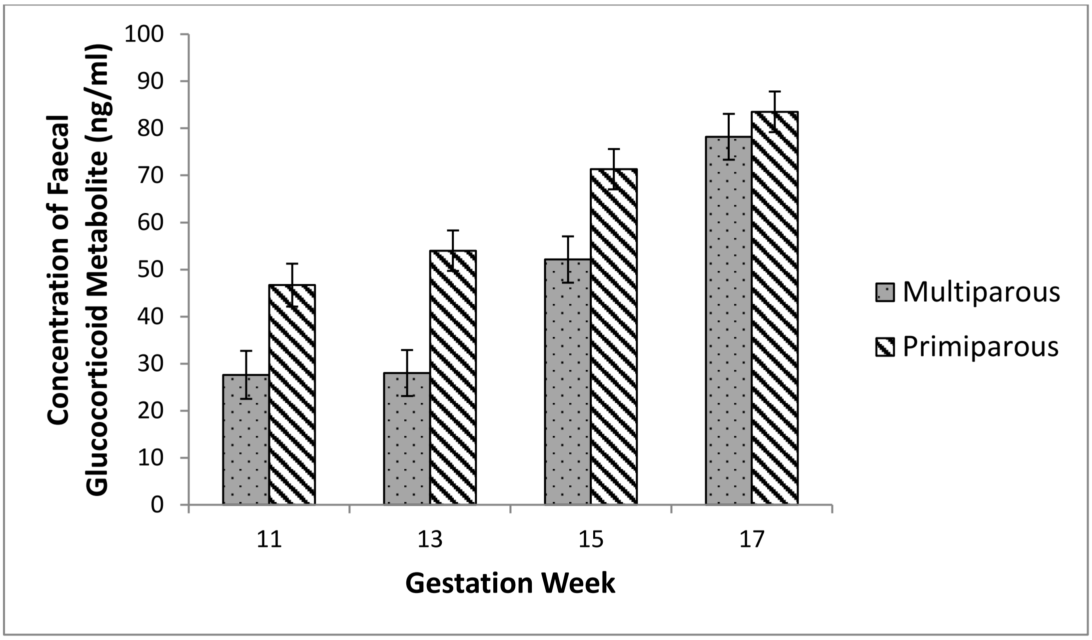

Figure 2.9. Concentration of FGM over the gestation weeks 11-17 for multiparous and primiparous ewes. Values are means (±SEM). Primiparous ewes had siginificanty higher concentration of FGM compared to multiparous ewes at week 11, 13, and 15 of gestation (P = 0.014).

2.3.5 Plasma oestradiol and progesterone

Treatment and parity as well as gestation week had no effect on the concentration of oestradiol and progesterone throughout the experiment (Table 2.7).

Table 2.7. Mean concentration of oestradiol (with CI) and progesterone (with SEM) recorded in pregnant ewes based on treatment groups, parity and gestation week.

	Oestradiol (pg/ml)	Progesterone (ng/ml)
Treatment		
Control	245.47 (198.6-303.5)	44.08 (3.45)
RS-Mix	243.78 (194.5-305.5)	42.31 (3.63)
	$F_{1,9.8}$ = 0.00, P = 0.97	$F_{1,11.7}$ = 0.14, P = 0.719
Parity		
Multiparous	252.35 (208.8-305.0)	42.92 (3.2)
Primiparous	236.59 (197.5-283.4)	43.47 (3.07)
	$F_{1,59.3}$ = 0.37, P = 0.547	$F_{1,164.2}$ = 0.09, P = 0.768

Gestation week		
13	237.68 (199.3-283.4)	39.31 (4.35)
15	254.68 (214.5-302.3)	44.39 (4.23)
17	240.99 (203.0-286.1)	45.89 (4.46)
	$F_{2,109.1}$ = 0.68, P = 0.507	$F_{2,66.4}$ = 0.56, P = 0.573

2.4 Discussion

The results obtained in this current study indicate a mild stress may be experienced by the RS-Mix ewes as they displayed higher levels of aggressive behaviour at week 14 and 16 of gestation. However, parity differences seemed to have more impact on the body weight change, behavioural, and physiological aspects on the ewes during gestation.

Parity seems to have a considerable impact on weight gain where primiparous ewes showed lower weight gain than multiparous ewes at week 13 and 15 of gestation. The primiparous ewes were first introduced to indoor environment during this study while multiparous ewes had been exposed to living indoors during late pregnancy until a few days after lambing in the previous year. A study investigating sheep transferred from pasture to indoor crates recorded withdrawal behaviour in weeks 2 and 3 of confinement (Fordham et al., 1991). Exposure to confinement in addition to a novel environment may also lead to disruption of feeding behaviour. Sheep have been observed to refuse feeding on novel food in an unfamiliar location and consumed more familiar aversive food which they have been conditioned to avoid prior to relocation (Burritt & Provenza, 1997). These animals might display what is called as ‘neophobia’, which is also referred to as ‘shy feeder’ (Savage et al., 2008), which is more pronounced in unfamiliar than familiar environments (Burritt & Provenza, 1997). Feeding behaviour observed by scan sampling in this study showed that Multiparous ewes were seen to feed more compared to Primiparous ewes. Therefore, besides not having adjusted to being housed indoor for the first time, it may also be possible that the low weight gain achieved by the Primiparous ewes was due to the competition with multiparous ewes for feeding space at the hay rack although this parameter was not especially recorded in this current study. Special attention should be given to the weight loss or minimum weight gain on primiparous ewes in gestation since it has been demonstrated that ewes exposed to low nutrition in mid gestation gave birth to low birth-weight in lambs (Muñoz et al., 2009; Rooke et al., 2010). It may also cause an adverse effect on the establishment of the ewe-lamb bond since lambs from under-nourished ewes took longer to suck and vocalised more while the ewes themselves showed reduced in expression of maternal behaviour at the same time which could eventually compromise lamb survival (Corner et al., 2010; Dwyer et al., 2003)

Higher total aggressive interactions during feeding of concentrates were recorded when the feeding space was restricted. However the difference between RS-Mix and Control groups was only significant during the first feedface observation at week 14 of gestation although RS-Mix ewes still displayed high aggressive behaviour at week 16 before it declined considerably at week 18 of gestation. The decrease in aggressive behaviour shown by RS-Mix ewes may be due to the progression of gestation which decreased overall activity probably as the foetus gets heavier. Marsden and Wood-Gush (1986) reported an increase of displacement from ewes with reduced space allowance per ewe. The number of displacements was also found to be high with reduced feeding space in ewes provided with hay (Bøe & Andersen, 2010). However, in this study, the proportion of free leave (voluntary leave) was not affected by treatment group or parity which indicates there was also no difference in performing displacements. In contrast, it was found that RS-Mix ewes showed significantly lower free join compared to Control ewes, indicating ewes from RS-Mix groups had to apply some form of physical effort on other ewes in order to access the feed due to smaller feeding space available. Besides displaying higher total aggression, multiparous ewes were more likely to display forced entry to get to the feed trough regardless the treatment even though Control group had twice the length of feed trough compared to RS-Mix group. The Department for Environment, Food & Rural UK (DEFRA) has recommended approximately 45 cm of trough space for lowland ewes to prevent competition and aggression which might be detrimental to sheep welfare (Department of Environment Food & Agriculture, 2002). The feeding space allowance recommended by DEFRA is more than that provided to RS-Mix ewes and far less than Control ewes in this study, but still higher aggression was observed in multiparous ewes which indicate that space may not be the reason for the high aggression. Several studies has reported that older domestic as well as wild sheep are more aggressive compared to younger individuals (Favre, Martin, & Festa-Bianchet, 2008; Gorecki & Dziwinska, 2014; Hass, 1991). However, in general, female sheep tend to interact less with other females and perform shorter bouts of aggressive behaviour compared to the rams (Fisher & Matthews, 2001). The ewes also rarely show clash, mount or threat-jump type of antagonistic behaviour unlike their male counterpart (Fisher & Matthews, 2001). The majority of aggressive behaviour performed by the ewes in this study was pushing other ewes during concentrate feeding at the feed trough in order to get more access to feed. The birth weight of lambs from ewes giving birth for the first time had been reported to be significantly less compared to second pregnancy (Gardner et al., 2007) which had also been seen in the study on human (Wilcox, Chang, & Johnson, 1996) and horse (Meirelles et al., 2017). This may be due to the greater blood volume expansion caused by increased vascularisation as a result from the first pregnancy which may promote a greater foetal growth in the following pregnancies (Gardner et al., 2007). As the uterine blood flow is a major regulator of transplacental foetal nutrient supply (Wallace et al., 2008), multiparous ewes may need to increase their nutrient uptake for their large foetal weight and for themselves which could explain the higher display of forced entry in accessing the concentrate feed.

Ewes were seen to spend more time ruminating at week 12 of gestation before this dramatically declined at week 14 regardless of treatment groups and parities. The ewes may have been displaying withdrawal behaviour as a result of moving to a new environment. Done-Currie et al. (1984) also reported a similar outcome where newly confined sheep were seen to ruminate more compared to long-term confined sheep. At week 18 of gestation, RS-Mix ewes displayed a significantly higher frequency of ruminating than Control ewes. This difference may have occurred by chance or ruminating may act as a coping mechanism to chronically stressed RS-Mix ewes. Since stereotypic behaviours (repetitive and functionless behaviour) are not often performed by ruminants including sheep (Lawrence & Rushen, 1993), it is postulated that rumination may play a role in alleviating the impact of stress condition in a similar ways to stereotypies (Broom & Fraser, 2007). In general, Primiparous ewes ruminated significantly more frequently than Multiparous ewes. This is consistent with the rest of the outcomes in this study, indicating Primiparous ewes may have had a more difficult time to adjust to the new environment, being pregnant for the first time as well as competing with Multiparous ewes in the same pen at close proximity compared to the pasture. Idling behaviour was negatively correlated with ruminating behaviour as the ewes displayed low frequency of idling in week 12 of gestation before increasing at week 14 and remain constant until the end of observation at week 18 of gestation. This could be an artefact of scan sampling as the increased of ruminating behaviour may decrease the display of other behaviour observed by scan sampling.

Concentration of faecal glucocorticoid metabolite (FGM) were found to be higher in Primiparous than Multiparous ewes in weeks 11, 13 and 15 of gestation, but no difference was found between treatment groups. The higher level of FGM concentration in Primiparous ewes may be due to the physiological alteration associated with first pregnancy, which may have been related to increased stress experienced in first time mother. Speculatively, it may also be a possibility that higher concentrations of FGM in primiparous ewes was due to an increased metabolic rate in preparing the body for first pregnancy. The parity difference in FGM however, disappears at week 17 of gestation. As in humans, late pregnancy is also associated with increase cortisol level in sheep as found in this present study regardless of the parity (Keller-Wood & Wood, 2008; McMillen, Thorburn, & Walker, 1987) and this may have masked subsequent signals of stress in primiparous ewes. Hild et al. (2011) reported a higher salivary cortisol in pregnant ewes after being handled aversively, but showed no difference between parities. This was perhaps due to the time of handling test and salivary collection which were conducted at late-pregnancy (beginning from week 5 before birth) which may have masked the stress response in primiparous ewes to prepare them for parturition. No difference between treatment for the concentration of FGM in this study demonstrates that different types of stressful event may produce different impacts on the behaviour and physiology of the animals (Blanchard et al., 2001). It has been shown that neuroendocrine response to stressors may be attenuated during pregnancy in many species including humans (Young & Rose, 2002). Therefore, ewes in RS-Mix group may have displayed hyporesponsiveness towards the stressors presented in this study, hence no significant differences between groups were seen. Hyporesponsiveness to stressors is important in pregnant mothers since it is hypothesized to offer a protective mechanism to the fetus. This is crucial as increased maternal hypothalamo-pituitary-adrenal (HPA) has been associated with behavioural and physiological alterations in the offspring (Coulon et al., 2011; Roussel-Huchette et al., 2008; Weinstock, 1997).

Concentration of plasma oestradiol and progesterone did not differ between treatments, parities and gestation stages. The concentration of plasma oestradiol in pregnant ewes should remain at low levels until around week 17 of gestation before increasing gradually, and will then rise rapidly a few days before parturition (Dwyer & Smith, 2008). Since the last blood sampling was conducted on the first day of week 17, the changes in concentration level of oestradiol could not be seen. On the other hand, the concentration of progesterone in the plasma which should be increasing gradually around week 12 of gestation (Bassett et al., 1969) did not show in the results obtained. However, very high intra- and inter-plate CV% were found in both oestradiol and progesterone assays. On most plates, the R_2 value obtained from the fitted standard curve were less than 0.8. Hence, the results on the concentration of plasma oestradiol & progesterone were deemed unreliable.

In conclusion, inadequacies of housing system may have caused mild stress to the animals as shown by higher frequency of aggression in RS-Mix ewes at the feeding trough. Ewes in RS-Mix group may have displayed stressor hyporesponsiveness or the housing conditions allocated to RS-Mix ewes may have been too mild to have an impact on the body weight, behavioural and physiological status of the pregnant ewes. On the other hand, primiparous ewes were the most affected by the housing condition as they have lower weight gain and higher concentration of FGM compared to multiparous ewes. The difference may be due to the exposure that Multiparous ewes had to indoor housing in their previous lambing as opposed to zero experience in primiparous ewes. Extra consideration should be given in setting up the housing system for gestating ewes, especially those pregnant for the first time to attenuate their stress to novel environments, which will improve the welfare of the ewes and eventually the outcome in the offspring.

3. The Effect of Housing Systems on Mother-Offspring Interaction from Birth to Lactation

3.1 Introduction

Maternal behaviour in sheep, which includes the interaction between ewes and their lambs, has been well-researched compared with other species (Dwyer, 2014). The maternal behaviour expressed by the ewes is crucial in ensuring the survival of the offspring in postnatal life. However, there are several factors that are known to alter or impair the expression of maternal behaviour in ewes. Among these factors are maternal experience (Dwyer & Lawrence, 1998, 2005), breed (Dwyer & Lawrence, 1998, 2000; Le Neindre et al., 1998; Ocak et al., 2013; Pickup & Dwyer, 2011), difficult parturition (Darwish & Ashmawy, 2011), as well as nutrition intake by the ewes during pregnancy (Dwyer et al., 2003).

The effect of stress experienced during gestation on the offspring has been widely investigated in various animals including farm animals such as pigs (Jarvis et al., 2006; Rutherford et al., 2014), sheep (Averós et al., 2015; Coulon et al., 2011; Coulon et al., 2015; Roussel et al., 2004) and cattle (Lay et al., 1997). However, the effect of gestational stress on the mother itself, which can lead to disruption of maternal behaviour towards her offspring, has not been widely researched. Studies on rats for example, have reported that stress experienced during gestation could alter postpartum maternal behaviour of the mother by reducing maternal care towards their offspring (Baker et al., 2008; Carini & Nephew, 2013). In sheep, the limited studies that have been conducted concerning the effect of stress on maternal care provided to the lambs have given contradictory results. For example, maternal behaviour at parturition of ewes that were exposed to various aversive events (such as isolation, mixing and transport) from around week 14 to the final week of gestation did not differ from control ewes (Coulon et al., 2014). Conversely, in a different study, ewes that were aversively handled during the final 5 weeks of pregnancy groomed their offspring longer at parturition compared to ewes which have been gently handled (Hild et al., 2011).

Maternal immunoglobulins play an important role in providing offspring with defence mechanisms to combat diseases during the neonatal period until a certain point when their body can produce their own protective level of immunity. A study conducted in Pakistan showed the relationship between serum immunoglobulin G (IgG) concentration and mortality of lambs where they found that surviving lambs had higher concentrations of immunoglobulins than those that died during the neonatal period (Ahmad et al., 2000). However, it was not known whether the low concentration of IgG found in the lambs was due to the low concentration of colostral IgG produced by the ewes or due to the impaired ability of the lambs to absorb IgG efficiently into their systems. Not many studies have been conducted to investigate the concentration of colostral IgG in sheep that relates to housing condition during gestation. Cows exposed to heat stress during pregnancy have been shown to have a lower mean concentration of IgG in their colostrum (Nardone et al., 1997) than unstressed cows. In another study on cows however, heat stress did not affect the concentration of IgG in the heifers' colostrum, but lower concentrations of serum IgG in the calves of heifers exposed to heat stress during gestation was recorded (Tao et al., 2012). As for ewes, one study has investigated the concentration of IgG in plasma during the late-lactating phase and drying-off period in relation to housing space allowance. In this study, ewes which have been subjected to high stocking density had lower concentration of plasma IgG compared to ewes housed in low stocking density (Caroprese et al., 2009). Parity has also been shown to affect the concentration of IgG in colostrum of ewes where colostral IgG concentration in primiparous ewes was higher than multiparous ewes (Higaki et al., 2013).

The aim of this chapter was to determine whether different housing systems experienced by the ewes at week 11 to week 18 of gestation could affect the behaviour of the ewes with regard to their offspring as well as the concentration of IgG in colostrum. In this chapter, it was hypothesised that a housing system that involves reduced space and social mixing would negatively affect maternal behaviour at 2 hours postpartum, the ability of the ewe to discriminate her offspring in a recognition test at

12 hours postpartum, and mother-offspring interactions during lactation in the field. In addition it was hypothesised that less experienced ewes would show reduced maternal care. Impaired concentration of IgG in colostrum may also be seen in these animals.

3.2 Materials and methods

3.2.1 Lambing and data collection

This study involved the same 71 ewes that were part of the study as described in Chapter 2. After the end of the experiment and observation on ewes during gestation, the ewes remained in the same pen and were left undisturbed until parturition. The RS-Mix pens were expanded to meet the dimensions of the control pens so that all 11 pens were the same size to provide more lambing space for the ewes. All ewes were provided with hay and water ad libitum until they were taken to the field at least 48 hours after lambing. The ewes lambed in April 2014 over a period of 18 days. Two observers were present in the shed 24 hours per day to conduct observations and tests, collect samples and assist the ewes where necessary. Intervention and assistance toward the ewes were kept at minimum and only given if the ewe had failed to progress through parturition in a certain period of time, which were: 1 hour after fluids were detected with no parts of the lamb showing, and/or 2 hours after parts of lamb of the lamb were seen at the vulva without any obvious progress being made (as previously described: Dwyer and Lawrence, (1998)). At 30 minutes postpartum, lambs were caught, their navels were dipped in iodine (to prevent infection) and rectal temperature was taken. In the case where the second born lamb (L2) was born within 30 minutes from the first born lamb (L1), application of iodine and measurement of rectal temperature were conducted 30 minutes post-partum of the second lamb. A coloured tape was looped around the right hind leg of L1 to allow identification of birth order for further observations and tests. When the lamb had dried, the tape loop was replaced by spraying the ewe number on both sides of the lamb’s body and a bar over the shoulder (for L1) and rump (for L2).

3.2.2 Observation at birth

In this study, birth is defined as the moment when the pelvis of the lamb has passed through the vulva. During lambing, the assistance given to the ewe/lamb and the birth presentation of lamb were scored according to the scoring system in Table 3.1.

Table 3.1. Descriptions of scores for assistance given and presentation of lamb seen during parturition.

Score	Descriptions
Assistance given	
0	No assistance provided, the ewe delivered the lamb unaided within 2 hours of beginning labour
1	Partially assisted (e.g. adjusting the presentation of lamb, before the ewe delivered the lamb unaided)
2	Lambed manually – the ewe was assisted to deliver the lamb until it was completely free of the ewe
3	Veterinary assistance required
Presentation	
1	Lamb is head first, with one foreleg forward and one foreleg back
2	Lamb is head first with both forelegs back
3	Back legs first
4	Breech – the lamb is backwards with all the legs extended towards the ewes head, often only the rump or tail can be felt
5	Two lambs together – both twins are present in the pelvic canal
6	Head back – the lamb has its foreleg extended but the head is rotated backwards

7	Caesarean – lambs were delivered by caesarean and no other presentation is recorded
8	Other (state)
9	Normal – lamb is head first with both forelegs extended and the nose lying along the legs

The position of the ewe at lambing (whether standing or lying) and whether the ewe gave birth during daylight or non-daylight hours were also recorded. For this study, 0610 until 2019 hr was considered as daylight hour while 2020 until 0609 was considered as non-daylight hour. However, during the non-daylight hours, artificial lights in the shed were switched on all the time and therefore the ewes maybe considered to be exposed to 24h light cycle. A High Definition video camera (Canon Legria HFM52, Japan) was placed on a tripod in front of the pen to continuously record the behaviours of both ewe and lamb starting from the birth of L1 up until 2 hours after L2 was born. This was complemented by a continuous 24-hour per day video recording using 14 EZ-Distributors video cameras linked to a Geovision digital video-recording system (Australia Pty Ltd) to store and view the footage. Cameras were mounted such that each pen was visible on the video record throughout the lambing period. Vocalisation made by the ewes and both lambs were recorded live using a Psion Workabout handheld computer (Psion PLC, London, UK) for 30 minutes after the birth of L2 (T30), followed by three 10 minute observations every 20 minutes from 50 minutes after birth until L2 was 2 hours old (T120). The collection of behavioural and vocalisation data (Table 3.2) were recorded using The Observer Software (Noldus Information Technology, Netherlands). For the maternal behaviour expressed in the first 2 hours postpartum, behaviour recorded by using the video camera was analysed by The Observer Software (Noldus Information Technology, Netherlands) for data collection. However, in the case where the video recorded was not clear (due to various factors i.e. bad camera angle or observed animals being blocked by other ewes), behaviour recorded using Geovision was used to collect behavioural data using The Observer Software.

Table 3.2. Ethogram of ewe's and lamb's behaviour from birth until 2 hours postpartum

Behaviour	Definition
Ewe	
Standing	Ewe is standing on all four legs, body clear of the ground
Lying	Ewe's body is in contact with the ground
Grooming	Ewe licks or nibbles her lamb
Circling	Ewe move away and walks around the lamb when the lamb is in contact with udder or attempts to suck
Backing	Ewe moves backward as the lamb is in contact with udder or attempts to suck
Moving forward	Ewe moves forward as the lamb is in contact with udder or attempts to suck
Leaves	Ewe walks away from her lamb, facing away from it - with a distance of up to 1 body length
Withdraw	Ewe backs away (1+ steps) from her lamb whilst looking at the lamb
Present udder	Ewe tilts hindquarter down, may turn back leg out as lamb contacts udder region
Butts	Contact with lamb with the front head towards any part of the lamb body
Pushes	Ewe presses lamb down or away with head
Paws	Ewe raises and touches lamb with foreleg
Low pitched bleat	Vocalisation made with mouth closed. Rumbling sound.
High pitched bleat	'Baa' vocalisation, made with mouth open
Birth	L2 birth
Lamb	
First Stand	Lamb supporting its own weight on all 4 feet for at least 5 seconds
Reach the udder	Lamb standing and/or moves actively (ie. doesn't just end up there if ewe has turned) towards udder region, nudging ewe or with head within 10 cm of udder.
Unsuccessful Suck Attempt	Lamb with head under ewe in immediate vicinity of udder, prevented from sucking by ewe movement, or fails to get

	teat into its mouth, or with teat in its mouth for less than 5 seconds.
Sucks	Lamb with head under ewe, has teat in its mouth, making sucking movements of head or noises, may be wagging tail, usually standing still, can sometimes see swallowing movements, for at least 5 seconds.

3.2.3 Colostrum sampling and measurement

Colostrum samples were collected from each ewe two hours after the second lamb was born. The ewes and their lambs were moved from the lambing pen into a small pen (1 m^2) to ease the collection process. Colostrum samples were obtained manually from both teats which were then placed in a labelled 5 ml plastic storage tube. All the samples were then frozen at 20^{o}C immediately after sampling until further analysis of IgG concentration. After the sampling of colostrum, the ewes and their lambs were then moved to one of the post-partum pens located in the experimental shed.

Analyses were carried out using an Ovine IgG ELISA Test Kit (Biopanda Reagents, UK). The kit provided a 96 well microtitre plate coated with anti-ovine IgG antibodies. All samples were diluted to 1:1,000,000 with the assay diluent provided with the kit. Seven concentrations of ovine IgG standard were prepared (1000, 500, 250, 125, 62.5, 31.25 & 15.625 ng/ml) and added to appropriate wells (100 µl / well). The plates were also filled with blank contained only 500 µl of assay diluent, and positive control (200 ng/ml IgG standard) at the beginning and end of each plate. Two dilutions of pool (1:1000000 & 1:2000000), using colostrum samples from 12 ewes were also placed at the beginning and end of each plate, and the rest of the wells were filled with the diluted samples to be analysed.

The plates were then incubated at 37 ^{o}C for 25 minutes before being washed four times with wash buffer. 100 µl HRP-Antibody Conjugate (15 ml of anti-Ovine IgG antibody conjugated with HRP in stabilising buffer) was then added to each well and incubated again for another 25 minutes at 37 ^{o}C before washing five times with wash buffer. Plates were blotted on absorbent tissue to remove residual wash buffer before adding TMB substrate solution (15 ml of TMB and hydrogen peroxide in a buffer, 100 µl / well) until colour was developed at approximately eight minutes before stopping the reaction with 100 µl of stop solution (15 ml of 0.3M sulphuric acid). The optical density was then read at 450 nm on a Multiskan FC spectrophotometer (Thermo Scientific, UK) using SkanIT Software 2.5.1. Coefficient of variation (CV%) intra-plates and inter-plates were 9.2% and 19.5% respectively.

3.2.4 Ewe recognition of the lamb

At 12 hours post-partum, the ability of the ewe to recognise her lamb was tested with 60 ewes (Control: n=31 (16 primiparous and 15 multiparous); RS-Mix: n=29 (15 primiparous and 14 multiparous)). Ewes were only tested when there were suitable sets of twin lambs available at the time of testing to be used as alien lambs (aged within 24 hours of test lambs). The testing pen (Figure 3.1) which was located at the front area of the experimental shed consisted of 3 similar size pens ($1m^2$): 1) ewe starting pen, 2) pen for own lambs, and 3) pen for alien lambs. The walls of the starting pen were covered with opaque material to prevent the ewe from seeing the two groups of lambs prior to the opening of the gate.

Before starting the test, the twin lambs of the tested ewe and a pair of alien lambs were carried from post-partum pen and were placed in the two small pens inside the testing arena. The side on which the alien and own lambs were placed was alternated at every test. The ewe was then led from the post-partum pen into the starting pen in the recognition test arena and was allowed at least 30 seconds to give her the opportunity to hear the bleats of the lambs. The test was recorded using a camcorder mounted on a tripod behind the starting pen and positioned as such that the entire test arena was visible. The test was 3 minutes long and started from when the gate of the starting pen was opened. The parameters recorded for this test were: (a) identity of first lambs approached, (b) latency to approach own lambs, (c) duration of time spent in the lambs' contact zone, and (d) duration of touching and looking towards a particular set of twins. Touching behaviour in this study was defined as when the muzzle of the ewe was in a very close range to the lambs performing some behaviours including sniffing, nosing and licking the lambs. Looking is defined as when the ewe's head and ears were directly oriented toward the lambs.

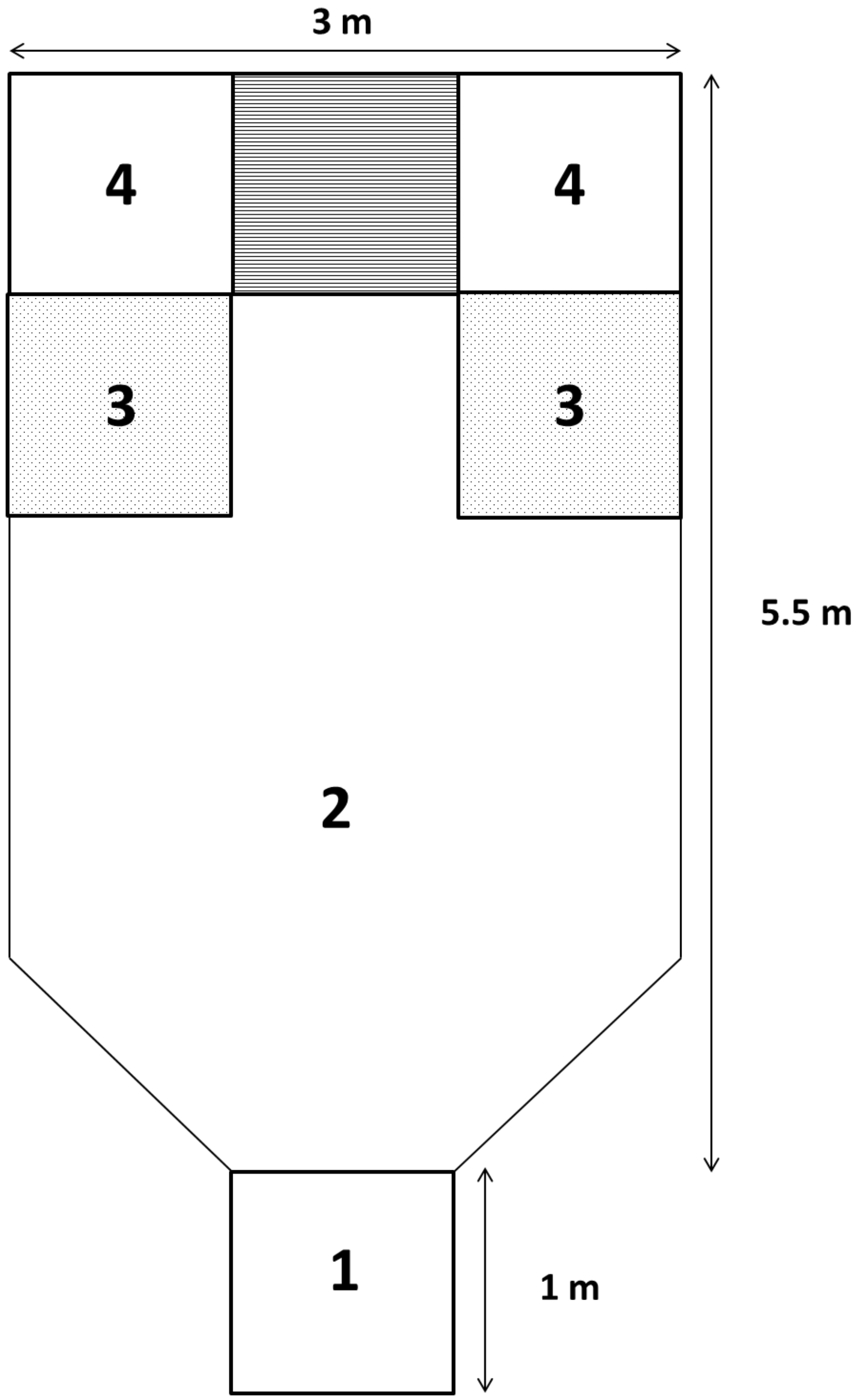

Figure 3.1. Representation of the pen used to test lambs' recognition by the mother. The numbered parts indicate the following: 1. Ewe starting point; 2. Neutral zone; 3. Contact zone with the lambs; 4. Pen for the lambs.

3.2.5 Behavioural observations during lactation

The ewes and their lambs were housed until approximately 3 days after birth (depending on the weather and health of the animals) before being taken to a field (5.03 ha) where all studied animals were kept as a single flock. Once in the field, the interactions between the ewe and lamb were further investigated when the lambs were between 3 days old until 6 weeks old using focal observation of a single ewe and her lambs and instantaneous scan sampling of the whole flock. For focal observation, 20 ewes were chosen to be observed (Control: n=10 (5 primiparous and 6 multiparous); RS-Mix: n=10 (4 primiparous and 5 multiparous)) whereas for scan sampling, all experimental ewes which still had both of their twins were observed (n = 62). Continuous focal observations on each individual ewe and their lambs were recorded for 15 minutes twice per week between 14:00 and 17:00 by a single observer using Observer data collection software loaded on a Psion Workabout handheld computer (Psion PLC, London, UK). In this focal observation, the detailed interactions between the ewe and her lambs, especially sucking interactions, were investigated.

Scan sampling was also used to obtain information on time budgets and ewe-lamb distance as well as distance between the ewes and their nearest neighbour (other ewes). Distances were estimated by taking ewe body length as approximately 1 meter. Scan samples were made either once or twice per day, for one or two days per week depending on when the focal observations were carried out. In the case when two scan observations were conducted in a day, the first was made in the morning (between 10:00 and 12:00) and the second in the afternoon (between 15:00 and 17:00). The ethogram for the behaviours recorded of both ewe and her lamb(s) is given in Table 3.3.

Table 3.3. Definition of ewe and lamb behaviours recorded on the field during (a) focal and scan observations, (b) focal observations only and (c) scan observation only from 1 week to 6 weeks postpartum.

Behaviour	Definition
(a) Focal and scan samples	
Ewe and lamb	
Stand	Standing with all four legs on the ground
Lie	Ewe's body in contact with the ground
Walk	Slow, leisurely movement, moving from one location to another
Ewe	
Vigilance (Head-up)	Standing still with head raised above the back, ears upright & forwards
Nose lamb	Ewe touches any part of the lamb with her muzzle
Lamb	
Successful suck	Lamb places its head under ewe and in contact with the udder for more than 5 s
(b) Focal samples	
Ewe	
Low pitched bleat	Vocalisation made with mouth closed/ rumble
High pitched bleat	'Baa' vocalisation, made with mouth open
Refuse suck attempt	Any movement that prevent a successful suck by lamb. E.g: moving away.
Terminate suck	Any movement that prevent the lamb from continuing to suck after a successful suck
Lamb	
Unsuccessful suck	Lamb places its head under ewe and in contact with the udder for less than 5s
Terminate suck	Any movement away from the ewe that ends a sucking bout
Bleat	Any vocalisation made by lamb

Approach ewe	Lamb moves directly towards the ewe to be at close proximity with the ewe (at least 1m from the ewe) from other location
Partial approach	Lamb approaching ewe but stop at least 5 m away before it reaches the ewe
Follow ewe	Walking or running not more than 1 m behind ewe in the same path
(c) Scan samples	
Ewe and lamb	
Run	Fast movement, moving from one place to another
Graze	Head down, biting, chewing, pulling grass and searching for food patches
Ruminate	Chewing/regurgitation of cud
Vocalise	Any type of bleating from ewe
Lamb	
Playing	Lamb displays any of these behaviours: gambolling (jumping & running), mounting & butting

3.2.6 Statistical analysis

In this chapter, the statistical analysis involved the data during parturition, maternal behaviour in the first 2 hours postpartum, concentration of IgG in colostrum, behaviours expressed in the recognition test, and the behaviour and spatial relationship between ewes and their lambs on the pastures during lactation. All analyses were conducted using GenStat 16th edition (Hemel Hempstead, UK) software. Data were checked for normality and transformed using log10 or square root transformation when necessary. For all transformed data, the mean are reported together with Confidence Interval (CI) instead of using Standard Error of Mean (SEM) as in untransformed data. Significance was considered to be $P < 0.05$, but some tendencies ($P < 0.1$) are also included. Where significant differences were found, post-hoc comparisons were made using Fishers' Least Square Differences (LSD) tests. Details on statistical analysis for each parameter tested are described below.

3.2.6.1 Parturition data and maternal behaviour

For the analysis in the first 2 hour post-partum, a total of 68 ewes were analysed. Three ewes were excluded from analysis; one ewe was not able to be observed due to technical problem with the video, while the other two ewes had a mummified foetus as their second lamb for which times of birth were not able to be determined.

3.2.6.1.1 Parturition data

Assistance given during parturition, presentation of lamb, birth position and the time of lambing (day or night) were analysed using General Linear Model (GLM) with a binomial function. Treatment group, parity and the interaction between the two were used as fixed effects. Due to a low number of partially assisted ewes, the three scores for assistance given at birth were simplified to only two scores: 0 for no assistance given and 1 which is a combination of lambing manually or assisted partially. A similar approach was applied to presentation of lamb at birth with score 0 for normal presentation and 1 for non-normal presentations.

3.2.6.1.2 Maternal behaviour during the first 2 hours post-partum

Maternal behaviour analysis commenced only after the birth of L2 except for the latency to groom the lambs which was recorded after the birth of both L1 and L2. From the 2 hours video recorded after the birth of L2, only the behaviour from the first 30 minutes (T30) and from 60-90 minutes (T90) after birth were analysed (except for vocalisations as have been explained in section 3.2.2). Since factors before and during birth could affect the behaviours of ewes and lambs (Table 3.4), an initial univariate analysis was conducted to identify the important variables to be fitted into the final model. For this purpose, one variable was used at a time with the appropriate response variable. Variables whose p-value ≤ 0.2 were selected to be fitted as fixed effects or covariates along with treatment and parity as an interaction in all analyses. However, since assistance given and presentation of the lamb during birth were found to be highly correlated in this study (Cramer's $V = 0.8$, $p < 0.001$), only assistance given during birth was used if both variables had a p-value of ≤ 0.2 in univariate analysis. Assisted ewes as well as ewes with non-normal lamb presentation during the birth of L2 were not included as factors in the analysis due to very low occurrence.

Table 3.4. List of variables used as fixed effect and covariates in the analysis of parturition data and maternal behaviour 2 hours post partum

Fixed effects	Covariates
Treatment	Interval between the birth of L1 and L2
Parity	Interval between sign of parturition (fluids seen) and L1 birth.
Time of birth (day or night)	
Assistance given to L1 during birth	
Presentation of L1 during birth	
Ewe position when giving birth to L1	
Ewe position when giving birth to L2	

As some behaviour was displayed infrequently, for a number of behaviours data were combined together into one category during analysis. Circling, forward and backward behaviours were combined into avoidance behaviour, whereas butt and push were combined into aggressive behaviour. The details for all analyses conducted for maternal behaviour displayed within 2 hours postpartum are presented in Table 3.5.

Table 3.5. Table of response variables analysed 2 hours post partum with the type of analysis, data transformation, fixed effects and covariates.

Response variables	Type of analysis and distribution	Transformation (if any)	Fixed effects and covariate (if any)
Grooming behaviour			
Latency to groom L1	GLM (normal)	Log 10	L1 birth assistance L1 birth position Interval from fluid to L1 birth
Latency to groom L2	GLM (normal)	Log 10	L2 birth position L1 presentation
Duration of grooming both lambs	Repeated measures		L1 presentation Interval from fluid to L1 birth Interval between L1 and L2 birth
Sucking behaviour			
Occurrence of suckling at T30	GLM (binomial)		Interval between L1 and L2 birth
Proportion of ewes suckled by lamb at T90	GLM (binomial)		L1 assistance Interval between L1 and L2 birth
Avoidance behaviour			
Avoidance at T30	GLM (binomial)		L1 assistance Interval between L1 and L2 birth
Avoidance at T90	GLM (binomial)		L1 assistance L2 birth position Time of birth

			Interval from fluid to L1 birth
Other behaviour			
Occurrence of aggressive behaviour	GLM (binomial)		L1 assistance L1 birth position L2 birth position
Occurrence of Pawing	GLM (binomial)		Interval from fluid to L1 birth
Vocalisation			
Comparison between the frequency of LPV and HPV displayed by ewes	Sign test		-
Frequency of LPV	GLMM (poisson)		Time of birth L2 birth position
Frequency of HPV	GLMM (poisson)		-

3.2.6.2 Recognition test

For the recognition test, treatment, parity and their interaction were fitted as fixed effects. All non-normal data were successfully transformed using Log 10 transformation. The difference in the time spent by ewes in contact zones of their own lambs and alien lambs were first compared using two samples t-test. GLM with normal distribution was then used to analyse all other data except for the identity of first lamb approached which assumed binomial distribution.

3.2.6.3 IgG concentration in colostrum

For the concentration of IgG in colostrum, treatment, parity and their interaction were fitted as fixed effects while plate was fitted as a random effect. Analysis was run using REML Linear Mixed Models function in Genstat.

3.2.6.4 Behavioural observation during lactation on the field

From the instantaneous scan sampling conducted, if more than one set of data were obtained for each ewe within the same week, the data were averaged according to the lamb age (by week). Due to infrequency of behaviour displayed, only lying, standing, idling, grazing and ruminating behaviour were analysed in ewes. Data for lambs were pooled together and lying, standing, idling, grazing as well as sucking behaviour were statistically analysed. For behavioural activity budget, analyses were performed using logistic regression in GLMM to model binomial proportion, whereas for ewe-lambs and ewe-ewe distance, the data underwent log 10 transformation and were analysed using REML Linear Mixed Models.

For the suckling behaviour data collected by focal sampling, the 6 weeks data were averaged over 2 weeks block (week 2, 4 and 6). Frequency of the ewes being suckled by lambs was analysed using GLMM with Poisson distribution while the suckling duration was analysed using REML Linear Mixed Model after underwent Log 10 transformation. For all analyses, lamb age (week), treatment, parity and their interaction were fitted as fixed effects while the ewe was fitted as random effect.

3.3 Results

3.3.1 Parturition

During parturition, parameters such as assistance given to the ewes, presentation of the lamb, posture while giving birth and time of parturition (daylight or non-daylight) were recorded (Table 3.6).

Table 3.6. The number (*n*) of ewes recorded for birth parameters during the parturition of both L1 and L2

Parameters	L1 birth (*n*)	L2 birth (*n*)
N = 68		
Assisted	22	4
Non-normal presentation	20	5
Give birth while lying	56	35
Give birth while standing	12	33
Give birth during daylight	49	-
Give birth during non-daylight	19	-

There were no differences between treatment group and parity in assistance given to ewes during the birth of both L1 and L2 or the presentation of both L1 and L2 (Table 3.7).

Table 3.7. Mean probabilities of assistance given during parturition (with CI) to Lamb 1 (L1) and Lamb 2 (L2) as well as the presentation observed in both lambs during parturition (with CI) based on treatment and parity.

	Assistance		Presentation	
	L1	L2	L1	L2
Treatment				
Control	0.30 (0.17-0.46)	0.24 (0.13-0.40)	0.27 (0.15-0.43)	0.27 (0.15-0.43)
RS-Mix	0.35 (0.21-0.53)	0.09 (0.03-0.25)	0.32 (0.18-0.50)	0.22 (0.11-0.40)
	Wald = 0.23, d.f. = 1, P = 0.629	Wald = 2.49, d.f. = 1, P = 0.115	Wald = 0.22, d.f. = 1, P = 0.642	Wald = 0.22, d.f. = 1, P = 0.641
Parity				
Multiparous	0.28 (0.15-0.46)	0.11 (0.04-0.27)	0.28 (0.15-0.46)	0.19 (0.09-0.36)
Primiparous	0.36 (0.22-0.53)	0.21 (0.10-0.38)	0.30 (0.18-0.47)	0.31 (0.18-0.47)
	Wald = 0.47, d.f. = 1, P = 0.492	Wald = 1.24, d.f. = 1, P = 0.266	Wald = 0.04, d.f. = 1, P = 0.838	Wald = 1.28, d.f. = 1, P = 0.258

However, ewes from RS-Mix group showed a tendency to give birth to L2 while standing compared to Control ewes which mostly gave birth to L2 while lying (mean probabilities of lying (CI range): Control: 0.624 (0.460 – 0.764), RS-Mix: 0.384 (0.232 – 0.563); Wald=3.78, d.f.=1, P = 0.052). Ewes from RS-Mix group also showed a tendency to give birth only during the daylight (24 out of 32 ewes) compared to Control group which was more balanced in giving birth during daylight (n = 20) and night time (n = 17) (mean probabilities (CI range): Control: 0.433 (0.285 – 0.595), RS-Mix: 0.224 (0.111 – 0.402); Wald= 3.20, d.f.= 1, P = 0.074).

3.3.2 Maternal behaviour within 2 hr post partum

3.3.2.1 Grooming behaviour

3.3.2.1.1 Latency to groom L1 and L2 after birth

There were no significant treatment differences in latency for ewes to groom their lamb after giving birth to either lamb (Table 3.8).

Table 3.8. Mean latency (seconds) of grooming Lamb 1 (L1) and Lamb 2 (L2) (with CI) as observed in ewes after parturition based on treatment groups.

	Lamb 1	Lamb 2
Treatment		
Control	68.71 (43.8-107.9)	50.70 (32.1-80.0)
RS-Mix	62.95 (39.5-100.2)	40.83 (25.7-65.0)
	Wald = 0.64, d.f. = 1, P = 0.428	Wald = 0.33, d.f. = 1, P = 0.578

However, ewes that were assisted during the birth process of L1, were slower to groom L1 after birth compared to ewes that were not assisted during the birth process (mean latency in seconds (CI range): Assisted: 80.35 (53.05-80.35), Not assisted: 28.05 (14.13-55.71); Wald = 7.95, d.f. = 1, P = 0.006). Ewes that gave birth to L1 while lying were also slower to groom L1 than ewes that gave birth while standing (Lying: 77.63 (55.09 – 109.39), Standing: 28.97 (13.57 – 61.84); Wald = 5.81, d.f. = 1, P = 0.019). Meanwhile, after the birth of L2, primiparous ewes were slower to groom the lamb (Multiparous: 24.38 seconds (13.93-42.66), Primiparous: 62.09 seconds (38.14-101.09); Wald=8.83, d.f.=1, P = 0.004). Similar to L1, ewes which gave birth to L2 while lying were also slower to groom compared to ewes which gave birth while standing (Lying: 72.44 (46.76 – 112.23), Standing: 27.42 (17.54 – 42.86); Wald = 9.26, d.f. = 1, P = 0.003).

3.3.2.1.2 Duration of grooming L1 and L2

There was no effect of treatment or parity on the duration of grooming L1 and L2 two hours post partum (Table 3.9).

Table 3.9. Mean duration (seconds) of grooming both Lamb 1 (L1) and Lamb 2 (L2) (with CI) observed in ewes after the birth of L2 based on treatment groups and parity.

	Mean duration (SEM)	Wald test
Treatment		
Control	381.9 (11.4)	$F_{1,69.5}$ = 0.13, P = 0.719
RS-Mix	376.1 (12.3)	
Parity		
Multiparous	386.2 (12.2)	$F_{1,69.5}$ = 0.80, P = 0.375
Primiparous	371.8 (11.5)	

However, ewes spent more time grooming during the first 30 minutes after L2 was born compared to during 60 – 90 minutes after L2 birth ($F_{5,113.8}$ = 53.91, P < 0.001; Figure 3.2) .

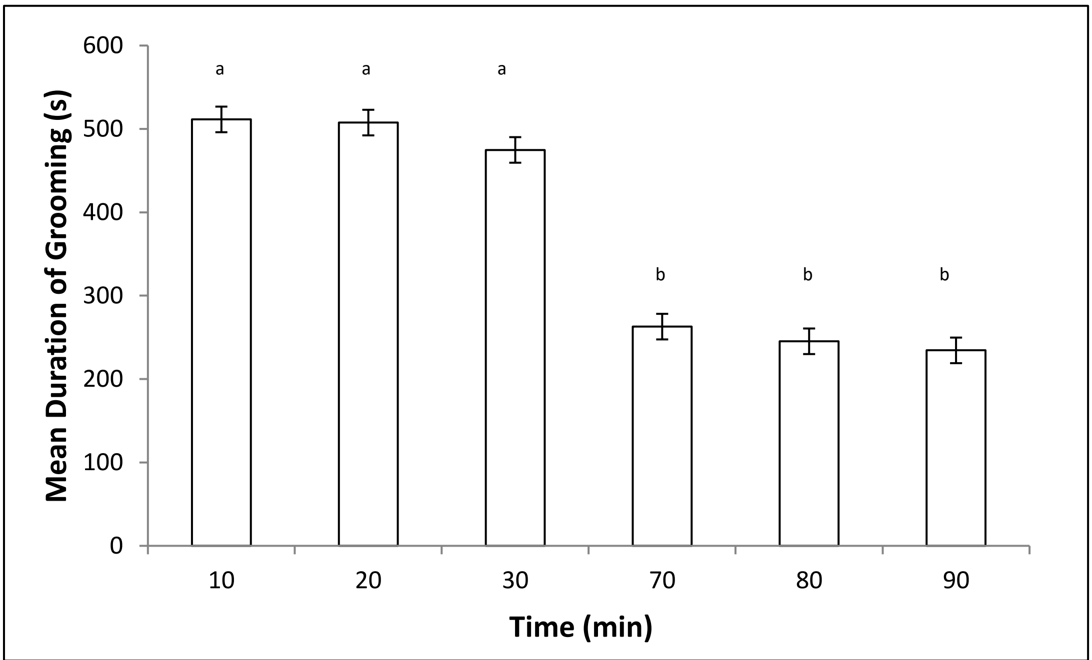

Figure 3.2. Duration of time spent grooming lambs by ewes over the 90 minutes observation period after the birth of Lamb 2. Values are mean duration with SEM as error bars. [ab] Different superscripts show significant differences at P<0.05.

3.3.2.2 Sucking behaviour

During the first 30 minutes after the birth of L2, only 36 out of 68 ewes (52.9%) observed had been suckled by their lamb(s). No effect of treatment or parity alone was found on the sucking behaviour. However the interaction between treatment and parity had an effect on the proportion of lamb sucking attempts that were successful. From post-hoc test conducted, Primiparous ewes from RS-Mix group and Multiparous ewes from Control group were significantly more likely to have been suckled compared to the other groups (Figure 3.3; Wald = 4.488, d.f. = 1, P = 0.034).

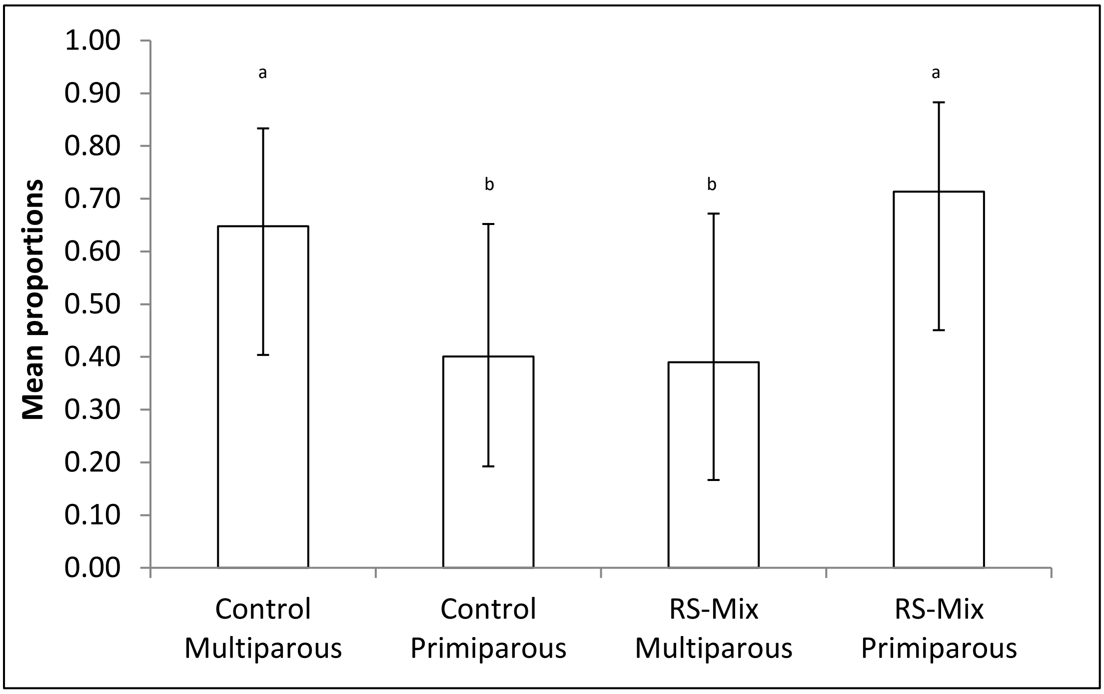

Figure 3.3. Proportion of ewes in each class that have been suckled by their lambs within 30 minutes after the birth of Lamb 2 (L2). Values are mean proportions with confidence interval (CI) as error bars. [ab] Different superscripts show significant differences between treatment*parity at P < 0.005 according to post hoc pair comparisons, using Fishers' Unprotected LSD.

Ewes with a longer duration between the birth of L1 and L2 have a higher occurrence to be suckled by their lambs during the first 30 minutes of L2 birth compared to ewes with shorter duration between L1 and L2 birth (Wald=7.336, d.f. = 1, P = 0.007).

During 60-90 minutes after L2 birth, no effect of treatment and parity on the proportion of ewe being suckled by L1 and L2 was found. However, ewes which gave birth to L1 without any assistance were more likely to have been suckled compared to ewes which were assisted during L1 birth (mean proportion (CI range): Not assisted: 0.35 (0.31 – 0.39), Assisted: 0.28 (0.24 – 0.33); Wald= 4.47, d.f.= 1, P = 0.034).

3.3.2.3 Avoidance behaviour

Avoidance behaviour performed by the ewe towards its lambs 30 minutes after the birth of L2 was found to be significantly affected by treatment and parity. RS-Mix ewes displayed a significantly higher proportion of avoidance behaviour when the lambs reached the udder compared to control group ewes (mean proportion of sucking attempts where avoidance behaviours were expressed (CI): RS-Mix: 0.11 (0.08 – 0.15), Control: 0.04 (0.02 – 0.07); Wald= 9.72, d.f.= 1, P = 0.002). Primiparous ewes performed a higher proportion of avoidance behaviour towards the lambs compared to multiparous ewes (Multiparous: 0.05 (0.02 – 0.07), Primiparous: 0.11 (0.07 – 0.14); Wald= 6.77, d.f.= 1, P = 0.009). In addition, ewes with a shorter duration between L1 and L2 birth also displayed higher proportion of avoidance behaviour when the lambs reached the udder compared to ewes with a longer duration between L1 and L2 birth (0.89 (0.82-0.93); Wald= 7.80, d.f.= 1, P = 0.005).

During 60-90 minutes after L2 was born, no effect of treatment was seen to influence the proportion of avoidance behaviour displayed by the ewes (Control: 0.10 (0.08 – 0.13), RS-Mix: 0.14 (0.11 – 0.19); Wald= 3.22, d.f.= 1, P = 0.073). However, as in the first 30 minutes after L2 birth, parity had a significant effect on avoidance behaviour with primiparous ewes having a higher proportion of avoidance behaviours when the lambs reached the udder compared to multiparous ewes (Multiparous: 0.09 (0.06 – 0.11), Primiparous: 0.16 (0.13 – 0.19); Wald= 10.44, d.f.= 1, P = 0.001). Ewes which gave birth during the day also showed a higher proportion of avoidance behaviour compared to ewes giving birth at night time (Day: 0.15 (0.12 – 0.18), Night: 0.09 (0.05 – 0.12); Wald= 5.91, d.f.= 1, P = 0.015).

3.3.2.4 Aggressive behaviour

Twenty-five percent of ewes (17/68) displayed aggressive behaviour towards their lambs after L2 were born. However, treatment and parity did not have any effect on the aggressive behaviour displayed (Table 3.10).

Table 3.10. Mean proportion (with CI) of aggressive behaviour displayed by ewes towards their lambs during the first 30 minutes and from 60-90 minutes after Lamb 2 (L2) were born based on treatment groups and parity.

	Mean proportion (CI)	Wald test
Treatment		
Control	0.25 (0.13-0.42)	Wald= 0.02, d.f.= 1, P = 0.878
RS-Mix	0.23 (0.11-0.42)	
Parity		
Multiparous	0.28 (0.15-0.47)	Wald= 0.49, d.f.= 1, P = 0.483
Primiparous	0.21 (0.11-0.37)	

3.3.2.5 Pawing behaviour

Of 68 ewes, 16 (23.5%) showed pawing behaviour towards their lambs after L2 were born. However, there was no significant effect of treatment and parity on frequency of pawing behaviour performed (Table 3.11).

Table 3.11. Mean proportion (with CI) of pawing behaviour displayed by ewes towards their lambs during the first 30 minutes and from 60-90 minutes after Lamb 2 (L2) were born based on treatment groups and parity.

	Mean proportion (CI)	Wald test
Treatment		
Control	0.26 (0.12-0.46)	Wald= 0.92, d.f.= 1, P = 0.338

RS-Mix	0.16 (0.06-0.34)	
Parity		
Multiparous	0.26 (0.12-0.48)	Wald= 0.81, d.f.= 1, P = 0.368
Primiparous	0.16 (0.07-0.34)	

3.3.2.6 Ewe vocalisations

Overall, at 2 hour post partum (a total of 60 minutes of recorded vocalisations), ewes demonstrated a significantly higher frequency of low pitched vocalisation (LPV) compared to high pitched vocalisation (HPV) (Median (CI range): LPV: 242.0 (169.9-288.1), HPV: 52.5 (24.6-97.1); P<0.001). There was no effect of treatment group or parity on the frequency of LPV in ewes after the birth of L2 (Table 3.12).

Table 3.12. Frequency of low pitched vocalisation (LPV) displayed by ewes during the first 30 minutes (T30) and the combination of three 10 minutes observations every 20 minutes from 50-120 minutes (T120) after the birth of Lamb 2 (L2) based on treatment groups and parity.

	Mean frequency (CI)	Wald test
Treatment		
Control	87.97 (63.4-122.0)	$F_{1,53.3}$ = 0.10, P = 0.750
RS-Mix	95.30 (64.5-140.8)	
Parity		
Multiparous	88.27 (58.2-122.1)	$F_{1,54.7}$ = 0.48, P = 0.492
Primiparous	99.48 (71.0-139.4)	

There was also no treatment effect on HPV (mean frequency (CI range): Control: 15.23 (8.68 – 26.72), RS-Mix: 17.74 (8.92 – 35.30); $F_{1,51.6}$ = 0.16, P = 0.689), but primiparous ewes made significantly more HPV compared to multiparous ewes (mean frequency (CI range): Multiparous: 11.26 (5.66 – 22.40), Primiparous: 27.94 (15.92 – 49.03); $F_{1,54.7}$ = 5.71, P = 0.02). As for the effect of time, the ewes made significantly more LPV at T30 and HPV at T120 regardless of the treatment group and parity (Table 3.13).

Table 3.13. Frequency of LPV and HPV in 30 minutes (means and confidence interval (CI)) at two different time frames, T30 and T120.

	T30	T120	P-value
Low pitch vocalisation (LPV)	106.57 (81.17-139.97)	78.65 (59.54-103.89)	$F_{1,59.7}$ = 18.59, P < 0.001
High pitch vocalisation (HPV)	12.43 (7.42-20.81)	21.71 (13.04-36.15)	$F_{1,59.4}$ = 46.37, P < 0.001

3.3.3 Recognition test

3.3.3.1 Approaching own lambs first

There was no evidence that the ewes recognised their lambs from a distance with only 28 out of 60 (46.67%) ewes approaching their own lamb first once the gate to the arena was open during the recognition test. Treatment group and parity also had no effect on the probabilities of approaching own lambs first instead of alien lambs. However, the interaction between treatment and parity showed a tendency to affect the latency of approaching own lambs (Wald= 3.05, d.f.= 1, P = 0.086). From post-hoc comparison, primiparous ewes from RS-Mix group took significantly longer to approach their own lambs compared to other groups (Table 3.14).

Table 3.14. Mean latency (in seconds) of ewes to approach their own lambs.

Groups	Mean duration (s)	Confidence Interval (CI)
Multiparous Control	6.45^{a}	4.47-9.31
Multiparous RS-Mix	5.95^{a}	4.07-8.70
Primiparous Control	6.73^{a}	4.72-9.60
Primiparous RS-Mix	11.93^{b}	8.27-17.21

^{ab} Within the mean duration column, different superscripts show significant difference between groups according to post hoc pair comparisons, using Fishers' Unprotected LSD ($P < 0.03$).

3.3.3.2 Duration spent in contact zones

There was no treatment or parity effect on the total time spent by ewes in contact zones (both own lambs and alien) during the 3 minutes observation. Nevertheless, from paired sample t-test conducted, tall ewes spent significantly more time in their own lambs' contact zone compared to the alien contact zone regardless of the treatment group or parity (Mean duration in contact zones (SEM): Own lambs: 135.4 (3.4), Alien lambs: 16.7 (2.1); $t(59) = 29.9$, $P<0.001$). However, by using Generalized Linear Model (GLM), it was observed that primiparous ewes spent significantly more time in their own lambs' contact zone compared to multiparous ewes (mean duration in seconds (SEM): Multiparous: 128 (4.7), Primiparous: 142.2 (4.6); Wald= 4.64, d.f.= 1, $P = 0.034$). There was no effect of treatment and parity on the time spent in alien lamb zone or in the neutral zone.

3.3.3.3 Duration of looking and sniffing lambs

There was no effect of treatment or parity on the duration of the ewes looking at their own and alien lambs. However, there was a tendency for the interaction of parity and treatment to affect the time spent sniffing own lambs (Wald= 3.16, d.f.= 1, $P = 0.081$) with multiparous ewes from RS-Mix group spending the least time sniffing their own lambs from the post-hoc comparison (Table 3.15).

Table 3.15. Mean duration (in seconds) of ewes from different groups spending time sniffing their own lambs.

Groups	Mean duration (s)	SEM
Multiparous Control	53.87^{a}	5.25
Multiparous RS-Mix	35.88^{b}	5.43
Primiparous Control	52.8^{a}	5.08
Primiparous RS-Mix	53.49^{a}	5.25

^{ab} Within the mean duration column, different superscripts show significance difference between groups according to post hoc pair comparisons, using Fishers' Unprotected LSD ($P < 0.027$).

3.3.4 Concentration of IgG in colostrum

There was no effect of treatment and parity alone on the concentration of IgG in colostrum. However, there was a tendency for the interaction between treatment and parity to affect the concentration of colostrum IgG ($F_{1,56.3} = 3.59$, $P = 0.063$). From the post-hoc comparison conducted, multiparous ewes of RS-Mix group had a significantly higher concentration of IgG in their colostrum compared to primiparous ewes from RS-Mix group (Table 3.16).

Table 3.16. Mean concentration of colostrum IgG (ng/ml) collected from ewes at 2 hours post partum based on the interaction between treatment and parity.

Parity*Treatment	Concentration (ng/ml)	SEM
Multiparous Control	47.91^{ab}	4.97
Multiparous RS-Mix	59.31^{b}	4.94
Primiparous Control	48.3^{ab}	4.56
Primiparous RS-Mix	43.48^{a}	4.76

^{ab} Within the mean concentration column, different superscripts show significance difference between groups according to post hoc pair comparisons, using Fishers' Unprotected LSD ($P<0.035$)

3.3.5 Behavioural observations during lactation

3.3.5.1 Activity budget

There were no significant effects of treatment and parity on the proportion of observation on all behaviour displayed by ewes during lactation in the field (Table 3.17).

Table 3.17. The proportion of observation (with CI) where specific behaviours were displayed by ewes observed during scan sampling in the field (standing, lying, walking, idling, grazing and ruminating) by treatment and parity.

Behaviour	Treatment		Parity	
	Control	RS-Mix	Multiparous	Primiparous
Standing	0.61 (0.56-0.67)	0.61 (0.55-0.67)	0.59 (0.54-0.65)	0.63 (0.57-0.68)
	$F_{1,56.4}$ = 0.03, P = 0.874		$F_{1,56.9}$ = 0.79, P = 0.379	
Lying	0.35 (0.31-0.40)	0.34 (0.29-0.39)	0.36 (0.31-0.41)	0.33 (0.28-0.38)
	$F_{1,56.1}$ = 0.17, P = 0.679		$F_{1,56.7}$ = 1.07, P = 0.306	
Walking	0.02 (0.01-0.04)	0.03 (0.02-0.06)	0.03 (0.01-0.05)	0.03 (0.02-0.05)
	$F_{1,55.8}$ = 0.85, P = 0.361		$F_{1,55.4}$ = 0.01, P = 0.909	
Idling	0.22 (0.17-0.28)	0.22 (0.17-0.28)	0.23 (0.18-0.30)	0.21 (0.16-0.27)
	$F_{1,53.9}$ = 0.00, P = 0.987		$F_{1,54.1}$ = 0.25, P = 0.622	
Grazing	0.45 (0.40-0.51)	0.42 (0.36-0.49)	0.41 (0.35-0.47)	0.47 (0.41-0.53)
	$F_{1,54.4}$ = 0.48, P = 0.491		$F_{1,54.5}$ = 2.65, P = 0.109	
Ruminating	0.25 (0.21-0.30)	0.27 (0.22-0.33)	0.28 (0.23-0.34)	0.24 (0.20-0.30)
	$F_{1,57.4}$ = 0.43, P = 0.514		$F_{1,58.0}$ = 1.09, P = 0.300	

However, time after parturition had a significant effect on some of the behaviour displayed by the ewes during the field observation (Figure 3.4). Ewes spent more time in idling behaviour in the first week after parturition which then decreased until week 7 postpartum ($F_{6,223.9}$ = 17.53, P < 0.001). In contrast, ewes displayed less grazing behaviour during the first two weeks of parturition before grazing increased at week 3 and remained constant throughout the observations ($F_{6,226.4}$ = 7.56, P < 0.001). Ewes also displayed less ruminating only during the first week after parturition and ruminating started to increase on week 2 before it remained constant until week 7 postpartum ($F_{6,230.3}$ = 2.30, P = 0.036). However, there were no significant effects of treatment or parity on the behaviour of ewes during lactation on the pasture.

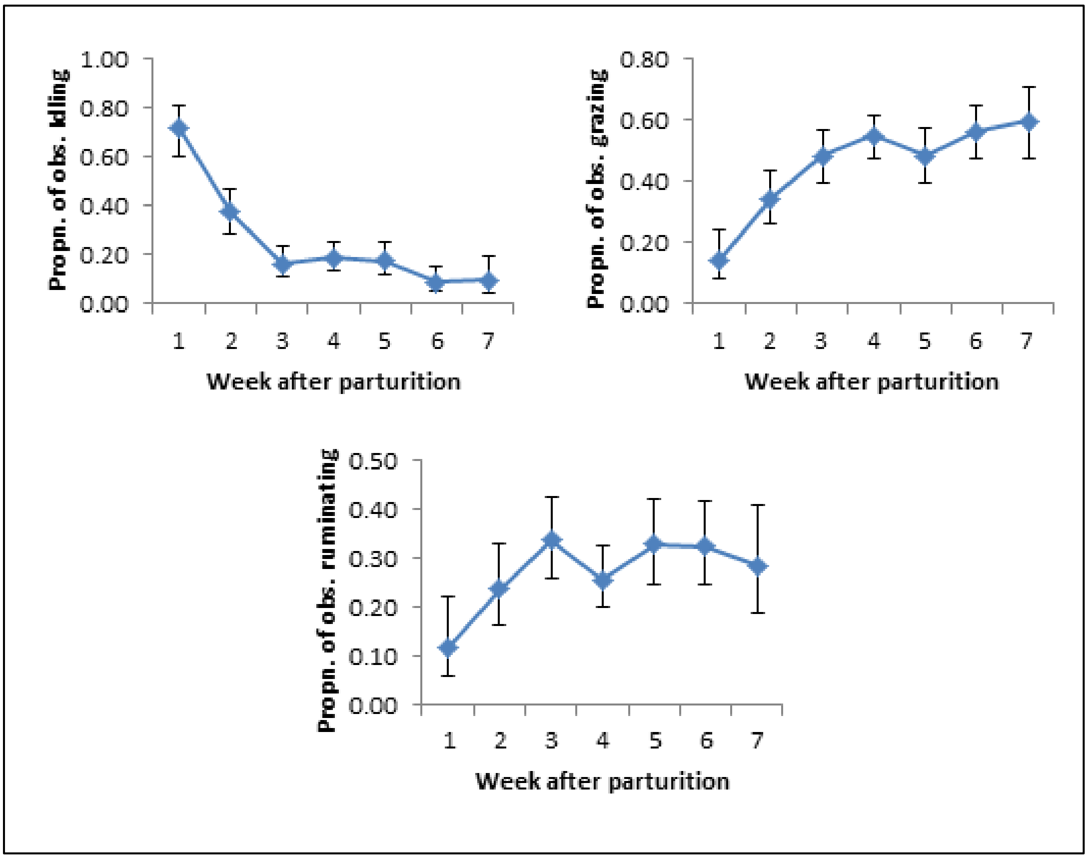

Figure 3.4. The proportion of observation (propn. of obs.) in specific behaviours displayed by ewes observed during scan sampling in the field (idling, grazing and ruminating) at different weeks after parturition. Values are mean proportion with confidence interval (CI) as error bars.

Lambs displayed a constant pattern of lying behaviour from week 1 to week 6 postpartum (Figure 3.5) before a significant decrease at week 7 postpartum ($F_{6,233.4}$ = 3.62, P = 0.002). This was in contrast to standing behaviour which was displayed less frequently during the first 6 weeks of observation and increased significantly during the final week of observation ($F_{6,235.0}$ = 3.81, P = 0.001). Lambs were more likely to be idle for the first five weeks postpartum which then decreased at week 6 ($F_{6,256.0}$ = 7.67, P < 0.001). They also grazed less for the first six weeks before it significantly increased at 7 weeks of age ($F_{6,234.0}$ = 5.89, P < 0.001).

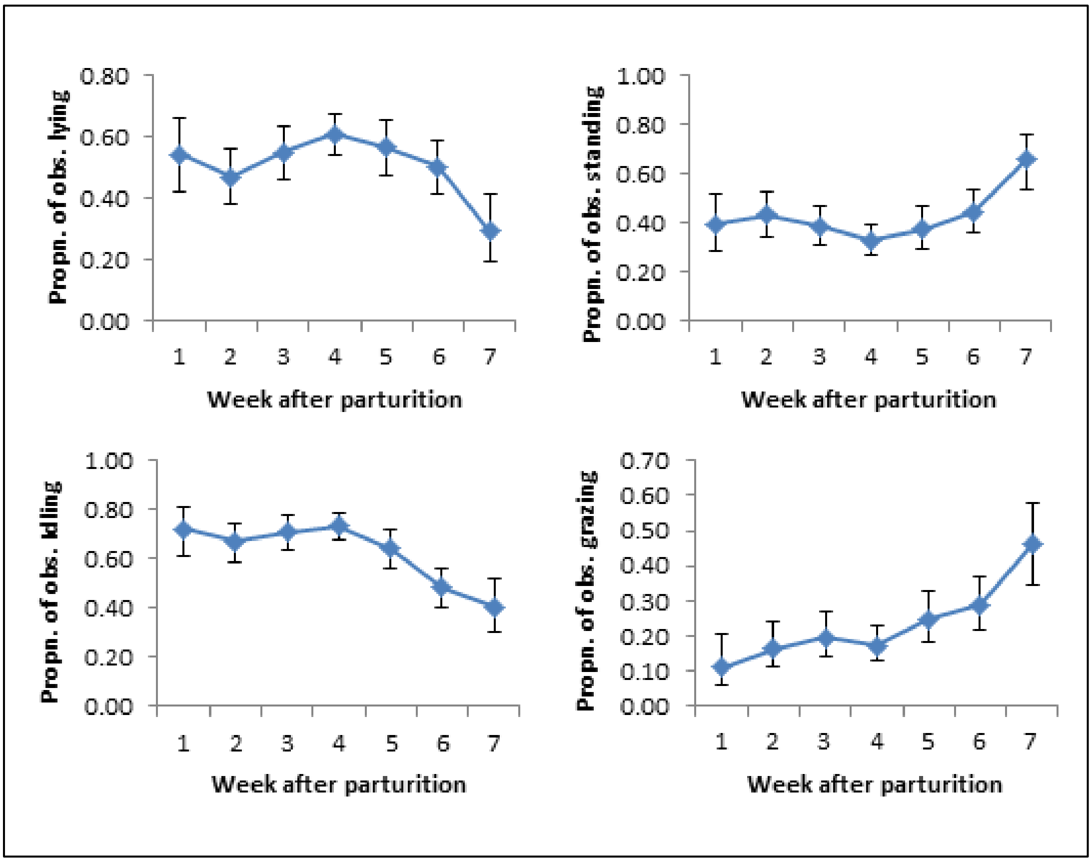

Figure 3.5. The proportion of observations where specific behaviours were displayed by lambs observed during scan sampling in the field (lying, standing, idling and grazing) at different ages (by week) after parturition. Confidence intervals for each behaviour were expressed as error bars.

However, sucking behaviour observed during this scan sampling was not affected by treatment, parity or age of lamb (Table 3.18). As for the effect of treatment, lambs from RS-Mix mothers displayed significantly less lying (and showed a tendency to display more standing behaviour compared to lambs from Control ewes. Lambs from multiparous ewes also showed a tendency to display more lying and idling behaviour compared to lambs from primiparous ewes (Table 3.18).

Table 3.18. The proportion of observation where specific behaviours were displayed by lambs observed during scan sampling in the field (standing, lying, idling, grazing, sucking) by treatment and parity.

Behaviour	Treatment		Parity	
	Control	RS-Mix	Multiparous	Primiparous
Standing	0.40 (0.36-0.46)	0.47 (0.41-0.52)	0.41 (0.36-0.46)	0.46 (0.41-0.51)
	$F_{1,55.5}$ = 3.09, P = 0.084		$F_{1,56.2}$ = 2.74, P = 0.103	
Lying	0.56 (0.51-0.61)	0.45 (0.40-0.51)	0.53 (0.48-0.59)	0.48 (0.42-0.53)
	$F_{1,56.4}$ = 8.32, P = 0.006		$F_{1,56.7}$ = 2.86, P = 0.096	
Idling	0.64 (0.60-0.68)	0.62 (0.57-0.66)	0.66 (0.61-0.70)	0.60 (0.55-0.65)
	$F_{1,256.0}$ = 0.62, P = 0.432		$F_{1,257.0}$ = 3.57, P = 0.06	
Grazing	0.21 (0.17-0.25)	0.23 (0.19-0.28)	0.20 (0.16-0.24)	0.24 (0.20-0.29)
	$F_{1,50.8}$ = 0.48, P = 0.491		$F_{1,51.7}$ = 2.56, P = 0.116	
Sucking	0.06 (0.04-0.09)	0.08 (0.05-0.11)	0.06 (0.04-0.09)	0.08 (0.05-0.11)
	$F_{1,256.0}$ = 1.18, P = 0.278		$F_{1,256.0}$ = 0.53, P = 0.467	

3.3.5.2 Spatial relationship between ewes, lambs and nearest neighbour

Ewes maintained a closer spatial relationship with their own lambs during the first two weeks after birth and the distance significantly increased from week three and remained on a plateau until week seven after birth ($F_{6,223.0}$ = 5.54, P < 0.001; Figure 3.6). Regardless of treatment groups, parity and age of lambs, the ewes stayed significantly closer to their own lambs compared to other neighbouring ewes (Median distance in m (CI range): Own lambs: 3.75 (3.00-4.375), Neighbouring ewe: 5.00 (4.75-5.5); P = 0.013).

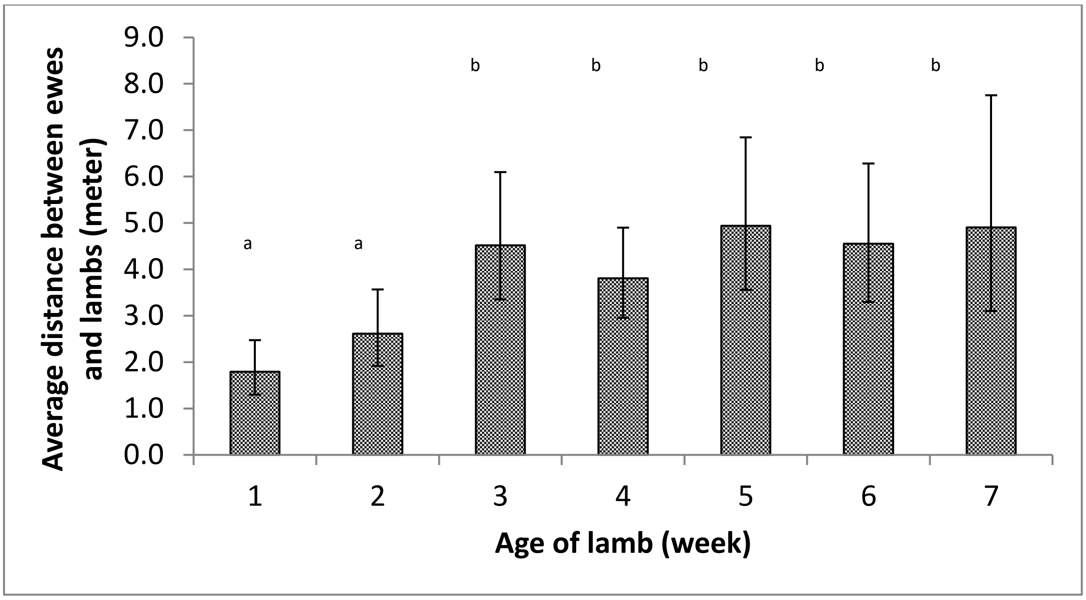

Figure 3.6. Mean of average distance between ewes and both her lambs in the field from the first week after parturition. Standard errors are shown as bars. [ab] Different superscripts show significant differences between groups according to post hoc pair comparisons, using Fishers' Unprotected LSD.

Interaction between treatment and parity had a tendency to affect the distance of a ewe with other neighbouring ewes ($F_{1,54.5}$ = 3.49, P = 0.067). From post-hoc comparisons, primiparous ewes of RS-Mix group kept a significantly greater distance from their neighbouring ewes compared to primiparous ewes of Control group and multiparous ewes from RS-Mix group (Table 3.19).

Table 3.19. Mean distance (meter) between observed ewes and their closest neighbouring ewe.

Groups	Mean distance (m)	CI
Multiparous Control	4.93^{ab}	4.39 – 5.53
Multiparous RS-Mix	4.66^{a}	4.04 – 5.35
Primiparous Control	4.73^{a}	4.18 – 5.36
Primiparous RS-Mix	5.70^{b}	4.98 – 6.51

[ab] Within the mean distance column, different superscripts show significant difference between groups according to post hoc pair comparisons, using Fishers' Unprotected LSD (P < 0.05).

3.3.5.3 Focal sampling on sucking interactions

There was no effect of treatment on the frequency and duration of lamb sucking bouts (Table 3.20). However, primiparous ewes displayed a significantly higher frequency of suckling their lambs in 15 minutes observation and a longer duration of sucking bouts compared to multiparous ewes (Table 3.20). Both frequency and duration of lamb sucking bouts also decreased significantly with lamb age regardless of the treatment and parity (Table 3.21).

Table 3.20. Treatment and parity difference in sucking behaviour (frequency in 15 minutes and sucking bout duration (s)) by the lambs during focal sampling in the field. Values are means with (CI).

	Sucking frequency (in 15 mins)	Sucking bout duration (s)
Treatment		
Control	0.77 (0.57-1.03)	12.22 (7.68-19.45)
RS-Mix	0.72 (0.53-0.98)	17.14 (10.67-27.53)
	$F_{1,12.0}$ = 0.08, P = 0.777	$F_{1,11.6}$ = 1.03, P = 0.332
Parity		
Multiparous	0.53 (0.39-0.71)	9.12 (5.73-14.52)
Primiparous	1.04 (0.77-1.41)	23.01 (14.26-37.13)
	$F_{1,12.0}$ = 9.8, P = 0.009	$F_{1,11.5}$ = 7.55, P = 0.018

Table 3.21. Difference in age of lamb on sucking behaviour (frequency in 15 minutes and sucking bout duration (s)) displayed by the lambs. Values are means with (CI).

	2 week	4 week	6 week	P-value
Sucking Frequency (in 15 mins)	1.19 (0.88-1.61)	0.69 (0.50-0.95)	0.50 (0.33-0.76)	$F_{2,22.5}$ = 6.81, P = 0.005

Sucking bout duration (s)	37.15 (23.34-59.14)	11.02 (6.65-18.26)	7.41 (3.91-14.07)	$F_{2,22.0}$ = 11.09, P < 0.001

3.4. Discussion

From the results, smaller space allowance and social mixing experienced during week 11 to 18 of gestation produced only minor effects on maternal behaviour. RS-Mix ewes were found to significantly prefer to give birth to L2 while standing instead of lying in contrast with Control ewes, and to display higher avoidance of lamb sucking attempts. For all other behaviours there were no overall effects of gestation treatment, although treatment interacted with parity for approaching and sniffing their own lambs in the recognition test with primiparous RS-Mix ewes being slower to approach than other groups, and Multiparous RS-Mix ewes spending less time sniffing their own lambs. Although the differences are minor, where significant differences have been found they do support the hypothesis that housing system in pregnancy has a negative impact on the expression of maternal behaviour.

Although there are very few studies that have been conducted looking at the position of ewes during parturition, in a long term study of 5 years observation involving more than 1500 ewes, two-thirds of the observed ewes gave birth standing or stood up immediately after birth (Alexander et al., 1993). This suggests that giving birth standing up might be a norm and therefore the high number of RS-Mix ewes which gave birth standing may have occurred by chance. However, the difference between Control and RS-Mix groups might also be due to an increased anxiety in RS-Mix ewes as exposure to stress had been shown to lead to heightened anxiety in both human and animal models (Cryan & Holmes, 2005; Leuner & Shors, 2013; Vyas, Pillai, & Chattarji, 2004). Therefore, since the cervix was already open from the birth of L1, RS-Mix ewes might chose not to lie down while giving birth to L2 as it might make them felt more vulnerable as the result of increased anxiety.

In a previous study, the time of parturition in ewes was evenly spread throughout the day (Arnold & Morgan, 1975) which was similar to the time of parturition of Control ewes in the present study. However, RS-Mix ewes significantly preferred to give birth during daylight instead of non-daylight hours (as mentioned in the methodology section 3.2.2, artificial lightings were provided al the time during non-daylight hours). The reason for this preference is unfortunately unknown. It might be since the RS-Mix ewes had heightened anxiety, the quietness of the non-daylight hour may make them more anxious and therefore prefer to give birth during daylight. It is also possible that the preference observed only occurred by chance which will need further research to clarify.

During maternal behaviour observations at 2 hours postpartum, RS-Mix ewes were less likely to cooperate with lamb sucking behaviour as they displayed a higher proportion of avoidance behaviour 30 minutes after the birth of L2 compared to Control ewes. A study by Coulon et al. (2014) produced an almost similar result where low-responsive ewes (less movement and vocalisation, and low cortisol concentration during isolation test prior to group assignment) exposed to various aversive events during gestation (GS) showed significantly higher rejection in lamb sucking attempts compared to high-responsive ewes from Control and GS groups. Even though no effect of treatment was seen in other aspects of maternal behaviour in this study, suckling the new born lambs might be one of the most difficult and requires more effort from the ewes and therefore, was the behaviour most disturbed by the housing system. One of the reasons may be due to the low supply of milk which has been found in ewes subjected to high stocking densities as well as being regrouped and relocated during gestation (Sevi, et al., 2001a; Sevi, et al., 1999). However, there was a high probability that less cooperation in lambs sucking attempt recorded in this study was due to the heightened anxiety in RS-Mix group as has been discussed above.

In this study, more differences in maternal behaviour related to parity rather than treatment were observed. Primiparous ewes took significantly longer to start grooming L2 after it was born and were less cooperative with lamb sucking attempts, which are in accordance with previous studies (Alexander et al., 1993; Dwyer & Lawrence, 1998, 2005). Although there was no significant effect of parity in the frequency of LPV made, primiparous ewes produced significantly more high-pitched vocalisation (HPV) compared to multiparous ewes. Previous studies have also demonstrated a high display of HPV by primiparous ewes in some breeds (Dwyer & Smith, 2008), and during a selectivity test at 2 hours postpartum (Keller et al., 2003). As in many species, ewes giving birth for the first time are not as competent as multiparous ewes in exhibiting maternal behaviour towards their offspring. However, from the results, no differences were seen in time spent grooming and LPV between parity and this suggests that primiparous ewes were as maternal as multiparous ewes. Therefore, it could be implied that as the presence of lambs was a novel experience for the primiparous ewes, the impairment in maternal behaviour observed in these ewes happened due to the fear and anxiety of coping with novel situations (Dwyer, 2014; Dwyer & Lawrence, 2000).

It may also be due to the greater anxiety in primiparous ewes that during recognition test at 12 hours postpartum, primiparous ewes spent significantly more time in their own lambs' contact zone compared to multiparous ewes. Multiparous ewes from RS-Mix group also displayed lower sniffing behaviour towards their lambs compared to other groups during the same time period. In a previous study, a significantly lower time spent near own lamb was recorded in primiparous ewes compared to multiparous ewes at 12 hours postpartum (Keller et al., 2003). However, there are studies in various species that support the result obtained in the present study where primiparous animals spent significantly more time in contact with their offspring (Guardini et al., 2015; Swanson & Campbell, 1979; Wischner et al., 2010). A similar result was found in a study on pigs where the females which had showed a neurobiological tendency to anxiety-related behaviour giving birth for the first time, were seen to spend longer visually attending to their piglets (Rutherford et al., 2014). One explanation may be that no gestational stress was applied to the ewes in Keller et al. (2003) study, unlike in this study and Rutherford et al., (2014) where social stressors were applied to the pregnant mothers. This suggests, even though there was only a mild effect of gestational stress observed during gestation, it may have been enough to activate the hypothalamic-pituitary-adrenal (HPA) axis and thus gave stronger impacts on the readily anxious primiparous ewes, which displayed more disturbed maternal behaviour. The concentration of faecal glucocorticoid metabolite (FGM) of primiparous ewes during gestation was significantly higher than multiparous ewes as shown in the previous chapter (Chapter 2). This may also be the reason why primiparous ewes from RS-Mix ewes showed a higher proportion of suckling their lambs 30 minutes after the birth of L2 and distanced themselves from neighbouring ewes during the observation on the field. Even though there was only a mild effect of housing on the RS-Mix ewes during gestation, it may have been sufficient in combination with parity, to result in these ewes displaying anxiety like behaviour and therefore displaying higher proportion of suckling their lambs and prefering to keep more distance with the neighbouring ewes to attend to their lambs. However, a higher proportion of suckling may also be due to the lower milk yield by primiparous ewes. It has been demonstrated in several studies that the mammary gland in primiparous females is not fully developed at first parturition and therefore, could result in lower rate of milk production compared to multiparous females (Fowler et al., 2014; Lang et al., 2011; Lang et al., 2012). Therefore, increased suckling time recorded may also be due to the low milk production in primiparous ewes. Primiparous ewes also displayed higher frequency of suckling their lambs and longer suckling bouts on the pastures during lactation. Higher frequency of suckling in primiparous ewes has also been seen in several other species such as in rhesus macaques (Gomendio, 1989) and grey seals (Lang et al., 2011). As have been discussed, this can also be due to the higher anxiety experienced by the primiparous mothers which may cause them to call to their lambs more frequently than multiparous ewes although vocalisation was not recorded in this observation.

During the recognition test at 12 hours post partum, primiparous ewes from RS-Mix were significantly slower in approaching their own lambs. No difference in latency to approach own lambs were observed between different parities in the study conducted by Keller et al. (2003). As has been mentioned above, the stressors that have been applied to the ewes in this study may have increased the activation of the HPA axis and therefore resulted in impairment of maternal behaviour in the primiparous ewes which can be seen in the previous chapter where higher concentration of FGM were recorded for primiparous ewes compared to multiparous ewes.

Ewes maintained a closer spatial relationship with their own lambs compared to neighbouring ewes from week 1 until week 7 after birth. However, from post-hoc tests, primiparous ewes from RS-Mix group kept a significantly larger distance from neighbouring ewes on the pastures compared to other groups. No experiments are known to have observed the effect of gestational stress on the spatial relationship of sheep on the field. Breed however, has been shown to affect the distance between other ewes in the flock in the field (Dwyer & Lawrence, 1999). Blackface ewes, which are more maternal and have a closer bond with their lambs, maintain a significantly larger spatial relationship with other conspecifics in the field compared to Suffolk ewes, which are not as maternal as Blackface (Dwyer & Lawrence, 1998). As all ewes in this study more or less displayed a similar pattern of maternal behaviour, it could be inferred that greater anxiety experienced by primiparous ewes from RS-Mix group, resulted in them spending more time with their lambs, and a larger distance from other ewes.

Interestingly, multiparous ewes from RS-Mix group had the highest concentration of IgG in colostrum compared to other groups. Studies in farm animals show that the concentration of IgG in colostrum increases with parity (Cabrera et al., 2012; Conneely et al., 2013; Gulliksen et al., 2008; Ha et al., 1986). However some studies in sheep and goat found that multiparous ewes had a lower concentration of colostrum IgG compared to primiparous ewes (Higaki et al., 2013) or showed no difference at all between parities (Alves et al., 2015; Argüello et. al., 2006). Cows and pigs subjected to heat stress produced a lower concentration of IgG in the colostrum (Merlot et al., 2013; Nardone et al., 1997). However, in studies investigating diet of ewes during pregnancy underfed ewes had the highest concentration of IgG in colostrum compared to well-fed ewes (O'Doherty & Crosby, 1996; Swanson et al., 2008). The mechanism for the altered concentration in IgG is unknown and inconclusive since there are not many studies which have been conducted on the effect of various type of housing stress on the concentration of IgG after lambing. However, this suggests that different type of stressors may produce different outcomes in the animals as each stressor might have its own pathway to elicit the response by the animals. This is thought-provoking and worth further investigation given the importance of IgG in providing immunity to the lamb and improving survival.

Besides treatment and parity, maternal behaviour at 2 hours postpartum could also be affected by what happened during parturition. Ewes giving birth while lying down had a significantly slower latency to groom L1 and L2 compared to ewes giving birth standing perhaps because they needed to spend some time to stand before they could then groom the lambs. A similar finding was recorded in ewes that were assisted during the birth of L1 where they took much longer to groom L1 since all of the assisted ewes (except two) gave birth while lying down. Assisted ewes during the birth of L1 also were being suckled the least by their lambs during 60-90 minutes after L2 birth. Difficult delivery has been associated with impaired maternal behaviour in ewes. These ewes are slower to begin grooming their lambs after parturition, spend less time grooming their lambs, make less LPV towards their own lambs and are less cooperative with lambs' sucking attempts (Darwish & Ashmawy, 2011; Dwyer et al., 2003). The negatively altered maternal behaviour may suggest that these ewes were affected by the difficulty they experienced while giving birth which may include the requirement for human intervention.

The longer the latency between the birth of L1 and L2, the greater the chance of the ewes being suckled within the first 30 minutes after the birth of L2. This was logical since most of the sucking was done by L1, and the longer the latency between births gave more time for L1 to develop greater coordination and to reach the udder and start sucking. Ewes which had a shorter latency between the birth of L1 and L2 displayed significantly more avoidance behaviour when the lambs went to the udder during the first 30 minutes after the birth of L2. This may have happened as the ewes may have not recovered from the birth of L1 and therefore felt some residual pain or stress when the lambs went to the udder to suck. The ewes might also be focusing on grooming L2 and so were less willingly to stand still for L1 to suck.

It is also an interesting finding in the present study at 60-90 minutes after the birth of L2, where ewes that gave birth to the lambs during the day showed a higher proportion of avoidance behaviour towards their lambs compared to the ewes giving birth at night. One of the factors that may contribute to this situation are the conditions in the shed. During the day, there were a lot of activities happening inside the shed (i.e. cleaning the shed, feeding the ewes, visits from veterinarian) as well as outside the shed. This may cause the new mothers to feel anxious which could disrupt the sucking attempts made by their lambs. In contrast, the conditions in the shed during the night were a lot calmer and quieter compared to during the day and so the ewes might be in a more relaxed state which eventually leads to less avoidance behaviour as the lambs attempt to suck.

Through the observation conducted in the field, no effect of treatment or parity was observed on the behaviour of the ewes. However, lambs from RS-Mix mothers displayed a higher proportion of time lying compared to lambs from Control mothers throughout the field observations. In a previous study, lambs from aversively treated ewes were seen to explore the environment less during human approach tests compared to lambs from gently treated ewes (Coulon et al., 2011). Lambs from ewes which were subjected to low space allowance during gestation and were separated from their mother 24 hours after birth displayed higher levels of immobility during novel arena tests (Averós et al., 2015). This may suggest that ewes subjected to inadequate housing systems during gestation may produce less vigorous or perhaps more fearful offspring which are less likely to explore their environment. However, lambs from undernourished ewes during gestation performed higher exploratory behaviour in the pen compared to higher intake ewes (Dwyer et al., 2003). This differing response in lamb behaviour suggests that the type of stressor experienced by the ewes may result in different outcomes for the lambs as well as for the ewes themselves and so must be considered.

In conclusion, this study has demonstrated that maternal behaviour of ewes and their interaction with their lambs vary according to treatment and parity. Although not many effects of treatment were observed in this present study, the higher avoidance displayed by RS-Mix ewes when the lambs attempted to suck suggested that the maternal behaviour of these ewes may have been impaired by the inadequacies of their housing system experienced during gestation. Primiparous ewes from RS-Mix group also displayed more anxiety-like behaviour than the rest of the ewes, which further strengthens the initial hypothesis that indoor housing condition and parity could alter the behaviour of the ewes not only during gestation, but also after parturition. Therefore, special consideration of the management system of the indoor environment should be taken into account when housing pregnant ewes to ensure the welfare of the ewes during gestation as well as for the lambs after lambing.

4. Effect of an alternative housing system, temperament and parity on the behaviour of ewes during gestation

4.1 Introduction

Among the findings recorded in Chapter 2, it was found that ewes in the RS-Mix group displayed significantly higher aggressive behaviour at the feedface on week 14 of gestation compared to control ewes. The display of aggressive behaviour in RS-Mix ewes then decreased from week 16 to 18 of gestation and showed no significant difference to Control ewes. The space allowance and the feeding space provided to Control ewes were almost double the size provided to RS-Mix ewes, which exceeds the maximum requirement for space allowance recommended by the Department for Environment, Food & Rural Affairs (DEFRA). However, ewes in Control group still displayed aggressive behaviour at the feedface during the provision of concentrate food. Koolhaas et al., (1999) explained that aggression exhibited by animals is part of their coping behaviour to environmental challenges in general. This implies that the ewes in the Control group with bigger space and feeding trough allowance may also have experienced some sort of stress which resulted in aggressive behaviour.

Since gestating ewes in the UK are usually kept indoors from mid gestation until parturition (Winter & Fitzpatrick, 2007), the introduction of an alternative system to reduce stressful conditions in the pregnant ewes and to provide the ewes with positive experiences while staying indoors should be considered. In addition to the physical conditions of the indoor housing, types of food may also affect the performance of gestating ewes. For example, ewes fed with silage showed significantly higher weight gain from mating to 7 days pre-partum compared to ewes fed with hay (Sormunen-Cristian & Jauhiainen, 2001). Silage is usually provided to the ewes *ad libitum* in order to avoid feed competition. Therefore, providing silage to the ewes in gestation may result in positive outcomes not only on the body weight, but also on body condition score, behaviour and physiology of the ewes.

In contrast to a good alternative system, ewes kept in a suboptimal housing system may also experience additional stressors. There is much more than space limitation that could additionally cause adverse effects on the welfare of the ewes in indoor housing. One example is exposure to dogs, which may happen regularly on farm. Ewes have been shown to be adversely affected by the presence of a dog compared to a human in an arena test (Beausoleil et al., 2005). A study investigating feeding behaviour in sheep also revealed that sheep avoided food in the presence of dog odour (Van Tien et al., 1999).

Unpredictability in management routines such as delay in food delivery could also occur in a farm setting (Destrez et al., 2013; Normando et al., 2013). Repeated occurrence of these unpredictable and uncontrollable events may result in the accumulation of negative emotional experiences in the animal (Destrez et al., 2013). In primates, unpredictable feeding schedules have been demonstrated to increase agonistic, abnormal and self-directed behaviour (Ulyan et al., 2006; Waitt & Buchanan-Smith, 2001). Delay in feeding has also been shown to affect behaviour in cows by increasing behavioural activities and movements before meals were provided (Normando et al., 2013), which suggest that the cows were disturbed by the feeding delay. A similar finding was also reported in female pigs presented with unpredictable feeding; where they displayed higher aggression which was believe to be induced by frustration (Carlstead, 1986).

This chapter is based on the same foundation as Chapter 2, which was to investigate the effect of housing system on the live weight, body condition score, behavioural and physiological aspects of gestating ewes. However, in this particular study, an alternative system which was predicted to promote positive experience in pregnant ewes was set up. The ewes in the alternative system were provided with *ad libitum* grass silage as well as higher space and feedface allowance, in an attempt to reduce aggression at feeding. A more negative housing system, which still attempted to replicate the normal on farm management of sheep, was also used. Ewes in the negative housing system were kept in pens with smaller space and feedface allowance, exposed to the presence of dog and experienced a delay in concentrate feeding schedule throughout the experiment. It was hypothesised that ewes in the Negative housing would display higher aggressive behaviour, lower weight gain and body condition score, and negatively altered physiological parameters such as the concentration of faecal glucocorticoid metabolites (FGM), plasma beta hydroxybutyrate (BHOB), oestradiol and progesterone. Haematological parameters particularly the Leukocyte profile were also hypothesised to be impaired in the Negative ewes. In contrast it was hypothesised that ewes housed in alternative housing would experience less stress which may be reflected in their behavioural and physiological parameters.

The effects of gestation week, parity and temperament on the ewes' outcomes were also investigated. Gestation week and parity had been shown to affect some parameters of the gestating ewes as presented in Chapter 2. Different temperament or individual variation in behaviour may elicit different responses to qualitatively different challenges (Beausoleil et al., 2012). In a study, sheep with a very high aversion to humans had significantly higher adrenocortical response to social isolation and transport compared to sheep not avoiding humans (Lankin, Stakan, & Naumenko, 1980). Therefore, it was hypothesised that differences in temperament of the ewes in this study may produce different responses in behavioural and physiological parameters during the experiment.

4.2 Material and methods

This study was conducted from early February until end of March 2015, at Woodhouselee Farm near Edinburgh. All procedures in this study were approved by the Scotland's Rural College (SRUC) Animal Experiments and Ethics Committee (approval ID: ED AE 04-2015) and were performed under UK Home Office license, following the regulations of the Animals (Scientific Procedures) Act 1986.

4.2.1 Animals

Similar to the study conducted in 2014 (Chapter 2), Scottish Mule ewes (Scottish Blackface X Blue-faced Leicester) in their first (primiparous) and second pregnancy (multiparous) were used in this study. All the ewes came from SRUC's own Woodhouslee flock and were naturally mated to a Suffolk rams in November 2015. The mating and ultrasonography scanning procedures were as described in Chapter 2 (2.2.1). From the ultrasound conducted in week 10 of gestation, 84 ewes pregnant with twins were chosen to be used in the experiment (primiparous, n = 43; multiparous, n = 41).

Ewes were then taken into the experimental shed before being weighed and their body condition was scored. Numbered tags from 1 to 84 were also placed around the neck of each ewe to ease the individual identification process. The ewes were placed in 12 pens with seven ewes per pen balancing for parity, weight, body condition score and temperament of individual ewes. The layout of the experimental shed with the arrangement of treatment pens is shown in Figure 4.1.

Ewes were vaccinated with Heptavac P Plus (Intervet, Ireland) at 17 weeks of gestation which was administered by subcutaneous injection in the lateral side of upper neck by an experienced technician. After data collection for the effects of housing in pregnancy was completed, ewes remained in the same pen until lambing.

4.2.1.1 Temperament test

Prior to allocation to treatment, and in order to determine temperament, the ewes were individually exposed to a 3 minute long isolation phase in a 2m^2 enclosure and 1 meter in height, and the floor was marked into nine equal size zones. The enclosure was covered all around with opaque material to prevent visual contact with other ewes in the shed. An EZ-Distributor video camera was positioned above the enclosure to record the movement of the ewes. Activity of the ewes and escape attempt were observed from the monitor of a Geovision digital video-recording system (Australia Pty Ltd) that was placed in a small shed next to the test arena. Activity was counted every time the ewes crossed a zone with at least one foot. Escape attempts were recorded when the ewe tried to cross over the test arena barrier by jumping. The test was ended if the ewes made at least three escape attempts or if they had jumped the barrier and escaped the arena. For the purpose of balancing the treatment group, activity per minute (APM) of all 84 ewes were calculated and categorised into two groups, High APM (HA) and Low APM (LA). 42 ewes with the highest APM were considered as HA ewes (average APM = 21.5) while 42 ewes with the least APM were considered as LA ewes (average APM = 4.3).

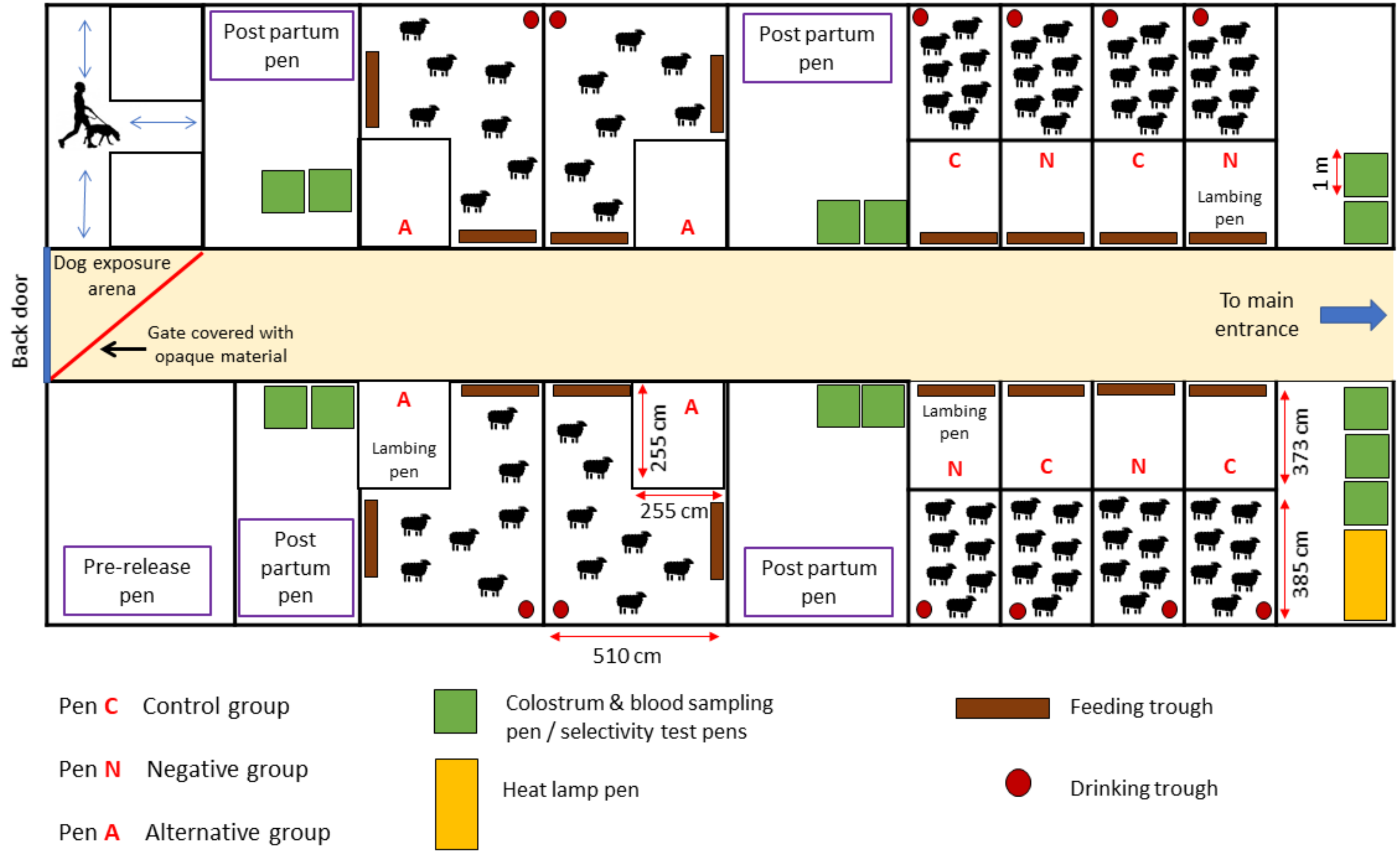

Figure 4.1. Layout of experimental shed during gestation and lambing.

4.2.2 Treatment groups

Ewes were divided into three treatment groups: 1) **Control**, 2) **Negative** and 3) **Alternative**, which differed in space allowance, feeding and exposure to additional stressor. As mentioned before, the treatment groups were balanced by parity and temperament group where each pen contained three or four ewes from different parities and temperament groups. The Control and Negative groups were provided with a space allowance of 1.28m^2/ewe and feedface allowance of 33cm per ewe. As for Alternative group, the ewes was provided with larger space allowance of 4.57m^2/ewe and feed allowance of 66cm per ewe, which was twice the size of feedface allowance provided to Control and Negative ewes.

The type of feed was also different depending on the treatment group. Control and Negative ewes were provided with hay *ad libitum* from the start of the experiment where the hay were filled into the hay rack every morning and late afternoon. Starting from 14 weeks of gestation, they were also provided with pelleted concentrate feed (20% crude protein, Davidsons Super Ewe Nut, Davidsons Brothers, Lanarkshire) which increased gradually until parturition (Table 4.1).

Alternative ewes however, were provided with grass silage (Dry Matter (DM) 251 g/kg; Protein 11.4 g/kg DM; Metabolisable Energy 11.4 MJ/kg DM) *ad libitum* which were placed in the feed troughs every morning from the start of the experiment at week 11 of gestation. From week 13 of gestation, the ewes were also supplemented with approximately 14g/ewe per day of vitamins and minerals (Norvite Farm Minerals for Cattle and Sheep, Norvite, Aberdeenshire) and starting from week 14 of gestation, they were also provided with 100g/ewe per day high quality vegetable protein made from soya (Sopralin: 46.5% crude protein, Trouw Nutrition, UK). The vitamins, minerals and protein were sprinkled on top of the grass silage in the feed troughs.

Table 4.1 Amount of concentrate feed provided to the ewes in Control and Negative groups throughout the experiment.

Gestation week	Amount of concentrate feed provided
Week 14	Concentrate feed were provided once a day starting with 1750gm per pen and adding 350gm everyday for each pen until it reached 3500gm per pen.
Week 15	3500gm per pen, once a day.
Week 16	Concentrate was provided twice a day for each pen with 2100gm in the morning and another 2100gm in the afternoon.
Week 17	Concentrate was provided twice a day for each pen with 2800gm in the morning and another 2800gm in the afternoon.
Week 18	Concentrate was provided twice a day for each pen with 3500gm in the morning and another 3500gm in the afternoon. This amount continued until lambing.

The normal time for placing concentrate feed in the feeding trough was at 0900 hr in the morning. However, besides normal feeding time, Negative ewes were also subjected to delayed feeding for 15, 30 or 60 minutes after normal time according to a predetermined schedule from week 13 of gestation until the end of experiment at week 18 of gestation. Control ewes were only subjected to predictable feeding times and did not experience any delay in feeding. During the time of concentrate feed, a radio with music was switched on and concentrates were placed in the feeding trough located in front of the lambing pen. The radio was used to indicate the concentrate feeding time for the Control and Negative groups at normal feeding time which may cause stressful situation for the ewes subjected to delayed feeding. After all feeding troughs in Control and Negative pens were filled, the gate of the pens was opened so the ewes could access the lambing pen and feed during this time, after which the radio was switched off. For Negative ewes when they were subjected to delayed feeding, the gate of the pens was kept closed until it was time for them to feed according to the time delay schedule. Ewes were allowed to be in the front pen until the feeding troughs were empty for a maximum of 30 minutes.

Ewes from the Negative group were also exposed to the presence of a dog once a week from week 11 to week 16 of gestation for a total of six times using six different dogs. The ewes were exposed to the dog in an arena located at the back end of the experimental shed (Figure 4.1). The gate and fences around the arena were covered with opaque materials to prevent the other ewes in the front part of the shed being able to see the dog and the exposure process. On the day of exposure, ewes from two Negative group pens were brought to the exposure arena and were placed in the two pens available in the arena. A dog would be brought into the arena using the back door by a handler where the dog was led in front of the pens in the exposure arena for 15 minutes. After 15 minutes, the ewes were returned to their experimental pens and the ewes from the other two Negative groups pens were then be brought to the exposure arena and underwent the same treatment.

4.2.3 Data Collection

4.2.3.1 Body weight and body condition score

Body weight and body condition score (BCS) of the ewes were first measured at week 9 of gestation on 23rd of January 2015 before the experiment started. During the experiment, body weight and BCS were measured again another four times. Both measurements were taken every Monday fortnightly at week 13, 15, 17 and 19 of gestation. Detailed methods in measuring the body weight and BCS of the ewes is as described in Chapter 2 (section 2.2.3).

4.2.3.2 Aggressive behaviour at feedface

Observation of aggressive behaviours at the feedface (refer to Chapter 2 (Table 2.2)) was conducted from Monday to Thursday in week 14, 16 and 18 of gestation. All pens were observed once every week except for the four pens from the Negative group which were observed twice a week as they were subjected to two feeding regimes; normal and delay feeding. As a result, four pens were observed each day beginning immediately after concentrates and silage were placed in the feeding trough during the morning feed. Observations were made using four camcorders (Canon Legria HFM52, Japan), each placed in front of the observed pen starting at 0930 hr every morning.

Prior to recording, the camcorder was positioned in front of the pen to make sure that it captured the whole feed trough and the feeding ewes.

4.2.3.3 Faecal sampling

Faecal samples were collected to measure the concentration of faecal glucocorticoid metabolites (FGM) in the ewes. The samples were collected from the rectum once a fortnight on Monday morning starting at 0900 hr in week 11, 13, 15 and 17 of gestation. Faecal samples that had been collected were then extracted in the lab and analysed for 11-oxoetiocholanolon cortisol metabolite. The details for sample collection, faecal extraction and enzyme immune-assay (EIA) method were as described in Chapter 2 (section 2.2.5). From the ELISA analysis, the coefficient of variation (CV%) of intra-plates and inter-plates were shown to be 2.4% and 8.45% respectively.

4.2.3.4 Blood sampling

Blood samples were collected to analyse the concentration of beta hydroxybutyrate (BHOB) in plasma, full haematology parameters as well as the concentration of pregnancy hormones (oestradiol and progesterone) throughout the experiment. Blood samples were collected via jugular venepuncture in weeks 13, 15, and 17 of gestation and were always sampled immediately after faecal samples were collected. The area where the blood was to be drawn was shaved beforehand for easier access of the jugular vein. The samples for BHOB and pregnancy hormones analysis were collected using heparinised vacutainers, while an EDTA vacutainer was used for analysing haematology parameters (BD Vacutainer®, Plymouth, UK).

After every two or three pens were sampled, the blood in heparinised vacutainers were centrifuged (Centrifuge 5702 R, Eppendorf, Germany) at 2500 rpm for 20 minutes in the experiment shed. The plasma obtained was then pipetted into three Eppendorf 1.5ml tubes. Two Eppendorf tubes to analyse oestradiol and progesterone hormones were frozen at -20 °C until further analysis in the lab. Another Eppendorf tube containing plasma was send to Analytical Service Department (ASD) of SRUC at Bush Estate to be analysed for the concentration of BHOB. The EDTA vacutainers containing blood were also sent to the ASD to be assayed for haematological parameters.

Plasma oestradiol and progesterone were measured using commercially available ELISA kits (Cusabio, Wuhan, China). Both kits provided a 96 wells microtiter plate which has been pre-coated with goat-anti-rabbit antibody. The details for ELISA analysis were as described in Chapter 2 (2.2.6). For the oestradiol analysis, coefficient of variation (CV%) of intra-plates and inter-plates were 20.4% and 37.9% respectively. As for the ELISA analysis of progesterone, coefficient of variation (CV%) of intra-plates and inter-plates were 14.9% and 34.9% respectively.

4.2.4 Statistical analysis

Of the 84 ewes, one ewe was removed from the analysis as it gave birth to triplets instead of twins. Gestation week, temperament categories, parity, treatment and their interactions were fitted as fixed effects, and individual ewe and pen were fitted as the random effects for all analysis.

For the purpose of analysis, the temperament categories of the ewes were divided into three based on the activity per minute (APM) rate obtained from temperament test conducted before the start of the experiment. Three categories were chosen instead of two due to the ambiguousness in the middle range value of the APM rate which may affect the interpretation of ewes' reactivity. The three new categories were highly reactive (HR) ewes, low reactive (LR) ewes and intermediate reactive (IR) ewes (Table 4.2). However, these three new categories were not reflected in the balancing purposes during pen allocations.

Table 4.2. The number of ewes in each temperament categories according to frequency of activity per minute.

Temperament categories	Frequency of activity per minute	Number of ewes in each group
LR	1.0 – 5.7	28
IR	6.0 – 13.7	27
HR	14.0 and above including Ewe 2 which escaped before the temperament test even started	28

All analyses were conducted using GenStat (16^{th} edition) software. Data were checked for normality and transformed using log10 or square root transformation when necessary. For all transformed data, the mean are reported together with Confidence Interval (CI) instead of using Standard Error of Mean (SEM) as in untransformed data. Where significant differences were found, post-hoc comparisons were made using Fishers' Least Square Differences (LSD) tests. Details on statistical analysis for each parameter tested are described below.

4.2.4.1 Body condition score (BCS) and body weight

For body condition score (BCS), two elements were analysed which are the absolute value of BCS and the overall changes in BCS which was derived by subtracting the final BCS recorded in week 19 of gestation with the BCS taken the first time during experiment which was on week 13 of gestation. The absolute BCS, overall BCS change and the average daily weight change data were analysed using Linear Mixed Model in REML analysis.

4.2.4.2 Concentration of beta hydroxybutyrate (BHOB)

Similar to BCS, the concentration of BHOB was analysed in two ways: 1) the absolute value and 2) the overall change of BHOB from week 13 to week 17 of gestation which was derived by subtracting the value recorded in week 17 with the value obtained in week 13 of gestation. Both absolute value and overall change value of BHOB were not normal and therefore were transformed using square root transformation before they were analysed using Linear Mixed Model in REML analysis.

4.2.4.3 Aggressive behaviour

Due to the low frequency of each different type of aggressive behaviour during the 30 min observation when concentrates were given, the data for push, butt, prod, mount, backpress, push-in, penetrate, displace and half displace behaviour were added together to make a new variable ‘total aggressive behaviour at the feedface’. In addition, the proportion of ‘free join’ (from the total of all type of joins to the feedface: join, push-in and penetrate) and ‘free leave’ (from the total of all type of leaving pen: leave voluntarily and being displaced) were also analysed.

Negative ewes were also analysed separately to compare the display of aggressive behaviour between normal and delayed feeding. The timings of the delay (15, 30 or 60 minutes) were combined into one delay category as no difference was found between the different timing delays. Aggressive behaviour at the feedface was analysed using a Generalized Linear Mixed Model (GLMM), fitting a Poisson distribution with a Logarithm function. The proportion of free join and free leaves which occurred at feedface were also analysed using a Generalized Linear Mixed Model (GLMM), fitting a binomial distribution with a Logit function.

4.2.4.4 Faecal glucocorticoid metabolite (FGM)

The data on the concentration of FGM was found to be non-normal and therefore it was transformed using log10 transformation. The data was then analysed using Linear Mixed Model in REML analysis.

4.2.4.5 Haematology

All haematology parameters were analysed using a Linear Mixed Model in REML. All blood parameters were normally distributed except neutrophil to lymphocyte ratio

(NLR), monocytes, eosinophils and platelets. These four parameters were normalised by a log 10 transformation prior to analysis.

4.2.4.6 Oestradiol and progesterone

Data for oestradiol, progesterone and ratio of oestradiol to progesterone (O:P ratio) were not normally distributed. Log10 transformation were then used to transform these data before they were analysed using Linear Mixed Model in REML analysis.

4.3 Results

4.3.1 Body condition score (BCS) and body weight of ewes

An interaction between treatment and gestation week was found to affect BCS throughout the experiment (Figure 4.2; $F_{6,237.0}$ = 3.60, P = 0.002). From post-hoc comparison, it was found that at week 17 of gestation, the BCS of Alternative ewes decreased significantly from week 15. In contrast, the BCS for Control and Negative groups did not change throughout the experiment.

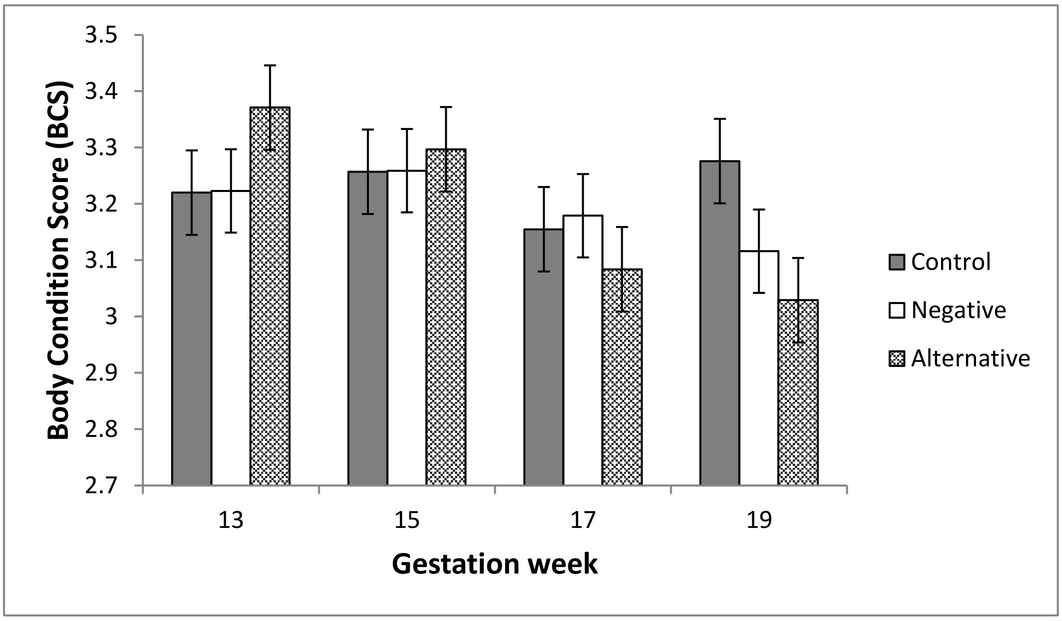

Figure 4.2. Body condition score (BCS) of ewes from Control, Negative and Alternative groups at week 13, 15, 17 and 19 of gestation. There was a significant decrease of BCS in Alternative ewes from week 15 to week 17 of gestation (P = 0.002).

Primiparous ewes were also found to have a significantly lower BCS compared to multiparous ewes throughout the experiment (mean BCS (SEM): Multiparous: 3.295 (0.05), Primiparous: 3.116 (0.049); $F_{1,70.4}$ = 6.96, P = 0.01). However, no temperament effect was found in the ewes' body condition score during the experiment (HR: 3.214 (0.06), IR: 3.207 (0.06), LR: 3.195 (0.06) ; $F_{2,73.0}$ = 0.03, P = 0.974).

The overall change in BCS between the final score taken in week 19 from week 13 of gestation was significantly affected by treatment and parity. BCS change in ewes from the Alternative group reduced from week 13 to week 19 which was significantly different compared to Control ewes whose BCS did not change (mean BCS changes (SE): Control: 0.056 (0.08), Negative: -0.105 (0.08), Alternative: -0.335 (0.08); $F_{2,8.9}$ = 5.96, P = 0.023). The change of BCS in primiparous ewes was also significantly different from multiparous ewes from week 13 to week 19 of gestation. Both groups showed a reduction in BCS throughout the experiment although primiparous ewes reduced the most (Multiparous: -0.016 (0.07), Primiparous: -0.240 (0.07); $F_{1,70.45}$ = 5.46, P = 0.022).

For average daily weight change, ewes from Alternative group had the lowest weight gain throughout the experiment (Figure 4.3; $F_{2,8.0}$ = 13.93, P = 0.002). Overall, little weight change was seen in all ewes between weeks 13 and 15 of gestation before it increased significantly in week 17 of gestation ($F_{3,243.0}$ = 20.03, P < 0.001).

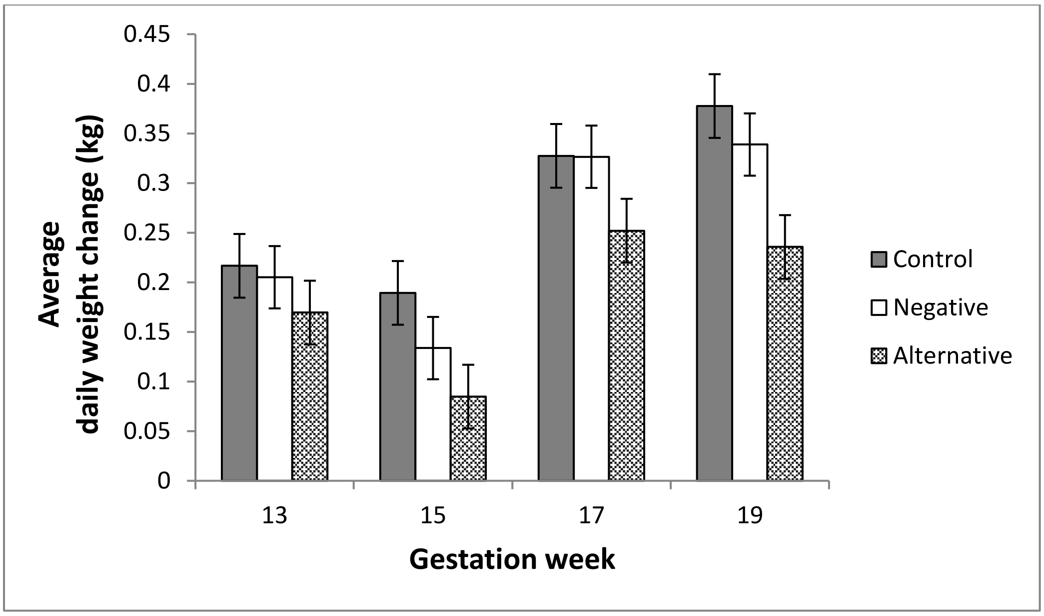

Figure 4.3. Average daily weight change (SEM) of ewes from Control, Negative and Alternative groups at week 13, 15, 17 and 19 of gestation. Alternative ewes had the least weight change throughout the experiment (P = 0.002). The daily weight change in all ewes also increased significantly from week 15 to week 17 of gestation (P < 0.001).

4.3.2 Concentration of beta hydroxybutyrate (BHOB)

There was a significant effect of treatment on the concentration of BHOB where ewes from the Alternative group had a lower BHOB concentration compared to Control and Negative groups (Table 4.3). At week 17 of gestation, the concentration of BHOB in ewes was significantly higher than in week 13 and 15 of gestation (Table 4.3). However, parity and temperament did not affect the concentration of BHOB in the ewes (Table 4.3).

Table 4.3. Mean concentration of BHOB (mmol/l) of ewes based on treatment groups and gestation week.

	BHOB (mmol/l)	Confidence Interval	P-value
Treatment			
Control	0.41[a]	0.36-0.47	$F_{2,88.4}$ = 73.26, P =
Negative	0.42[a]	0.37-0.48	0.005
Alternative	0.32[b]	0.27-0.36	
Gestation week			
Week 13	0.30[a]	0.27-0.34	$F_{2,175.0}$ = 73.26, P
Week 15	0.34[a]	0.30-0.37	< 0.001
Week 17	0.54[b]	0.49-0.59	
Parity			
Multiparous	0.35	(0.32-0.40)	$F_{1,87.9}$ = 2.71, P =
Primiparous	0.41	(0.36-0.45)	0.104
Temperament			
HR	0.37	(0.32-0.42)	$F_{2,87.8}$ = 0.20, P =
IR	0.38	(0.33-0.44)	0.819
LR	0.39	(0.34-0.45)	

[ab] Within the BHOB column, different superscripts show significant difference between treatment or gestation week according to post hoc pair comparisons, using Fishers' Unprotected LSD (p<0.05)

As for the change of BHOB concentration between week 13 to week 17 of gestation, post-hoc tests showed that primiparous ewes from Control and Negative group have higher BHOB concentration compared to primiparous ewes from Alternative group as well as from their multiparous counterparts in all three treatment groups (Figure 4.4; $F_{2,67.4}$ = 3.38, P = 0.04).

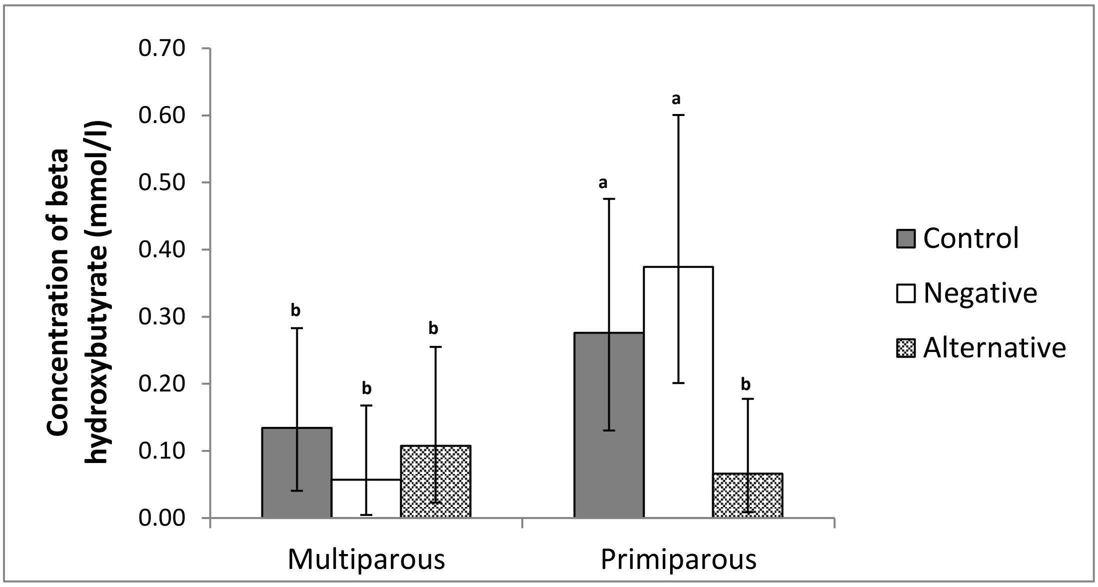

Figure 4.4. Changes in concentration of beta hydroxybutyrate (BHOB) in the plasma of multiparous and primiparous ewes from Control, Negative and Alternative groups from Week 13 to Week 17 with CI as error bars. [ab] different subscripts represent significant statistical differences within and between parity at P<0.05.

4.3.3 Aggressive behaviour at feedface

There were no parity (Multiparous: 1.70 (1.29-2.25), Primiparous: 1.64 (1.25-2.16); $F_{1,71.9}$ = 0.03, P = 0.859) and temperament (HR: 1.86 (1.34-2.58), IR: 1.72 (1.25-2.38), LR: 1.46 (1.04-2.04); $F_{2,74.3}$ = 0.20, P = 0.816) effects on the frequency of aggressive behaviours displayed by ewes in 30 minutes while feeding at the feeding trough. However, aggressive behaviour was affected by different treatment groups at different stages of gestation (Figure 4.5; $F_{4,161.9}$ = 2.77, P = 0.029). From post-hoc tests conducted, Control ewes displayed significantly higher aggressive behaviour compared to ewes in the Alternative group at week 14 of gestation. Control ewes also displayed significantly higher aggression at week 14 compared to week 16 of gestation. Ewes in the Negative group displayed high aggressive behaviour at week 14 which significantly decreased at week 16 before increasing again significantly at week 18 of gestation.

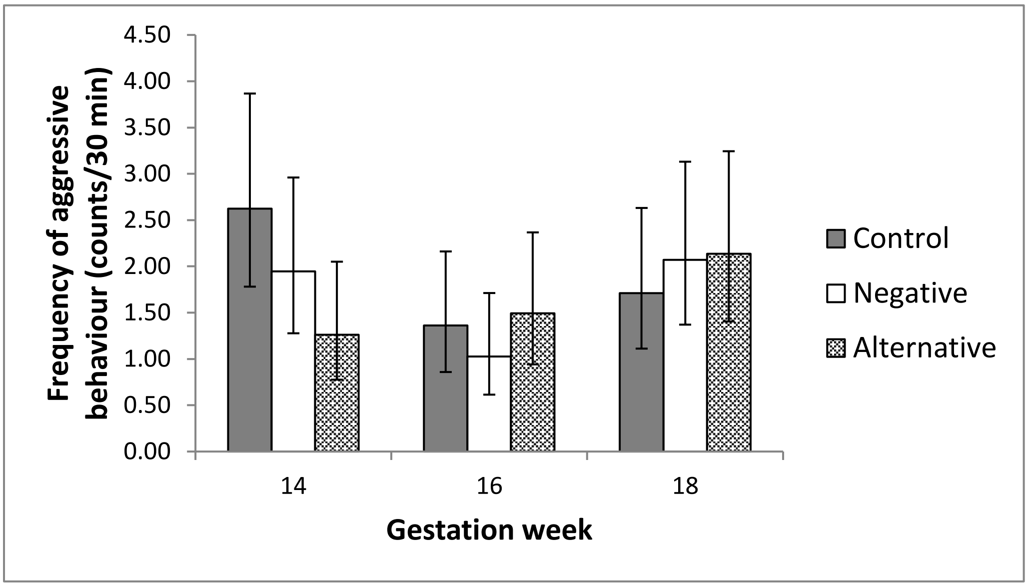

Figure 4.5. Frequency of aggressive behaviour displayed by pregnant ewes from different treatment groups at feeding trough at different gestation week. Data presented are means and CI as error bars. Control ewes displayed significantly higher aggressive behaviour at week 14 of gestation compared to other gestation weeks and other treatment groups (P = 0.029).

The frequency of aggressive behaviour was also affected by the interaction of temperament groups and week of gestation (Figure 4.6; $F_{4,161.9} = 2.68$, P = 0.033). From post-hoc test conducted, a significantly higher frequency of aggressive behaviour was displayed by low reactivity (LR) ewes at week 18 compared to week 14 and 16 of gestation. No difference in other temperament groups was found in relation to the week of gestation.

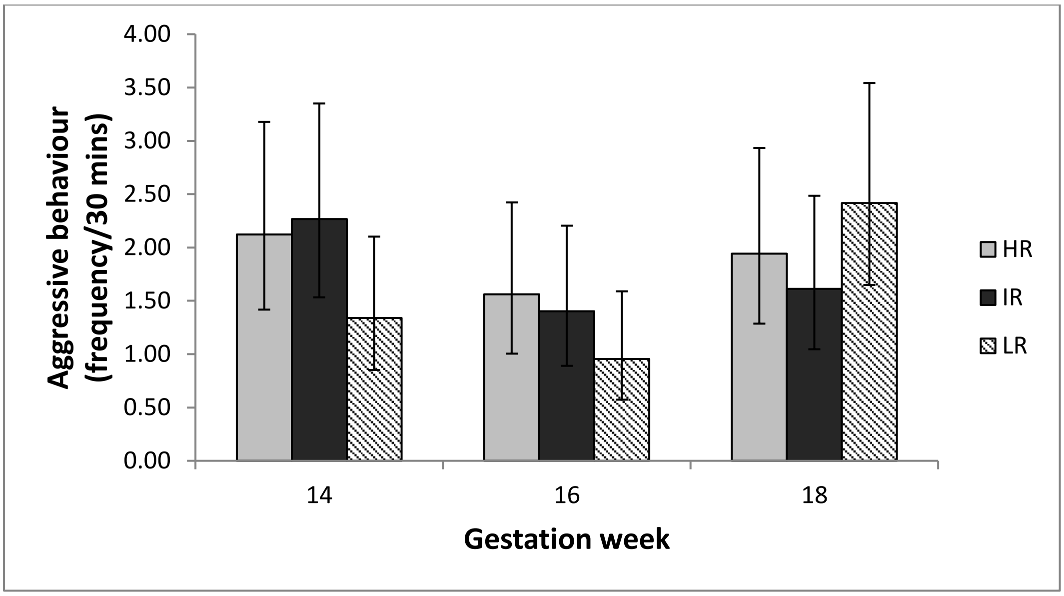

Figure 4.6. Frequency of aggressive behaviour displayed by pregnant ewes of different temperament classes (HR – high reactive, IR – intermediate reactive, LR – low reactive) at the feeding trough in different gestation weeks. Data presented are means of counts/30 min with CI as error bars. LR ewes displayed significantly higher aggressive behaviour at week 18 of gestation compared to HR and IR ewes (P=0.033).

For the Negative groups where two types of feeding regime were conducted (normal and delay feeding), the frequency of aggressive behaviour displayed was found to be affected by different feeding regime at different gestation weeks (Figure 4.7; $F_{2,135.6}$ = 4.17, P = 0.017). From post-hoc test conducted, ewes that were exposed to delayed feeding in week 14 of gestation displayed significantly higher aggressive behaviour compared to ewes that were fed normally. The frequency then decreased in week 16 and remained constant until week 18. For ewes that had been fed normally, aggressive behaviour displayed in week 16 of gestation was significantly lower than week 14 and week 18 of gestation (Figure 4.7). However, no parity (Multiparous: 2.00 (1.34-2.98), Primiparous: 1.30 (0.86-1.97); $F_{1,25.4}$ = 2.05, P = 0.165) and temperament (HR: 1.94 (1.19-3.16), IR: 1.64 (1.01-2.67), LR: 1.32 (0.80-2.16); $F_{2,24.4}$ = 0.57, P = 0.574) effects were found on the frequency of aggressive behaviour displayed by ewes in Negative group based on the type of feeding regime.

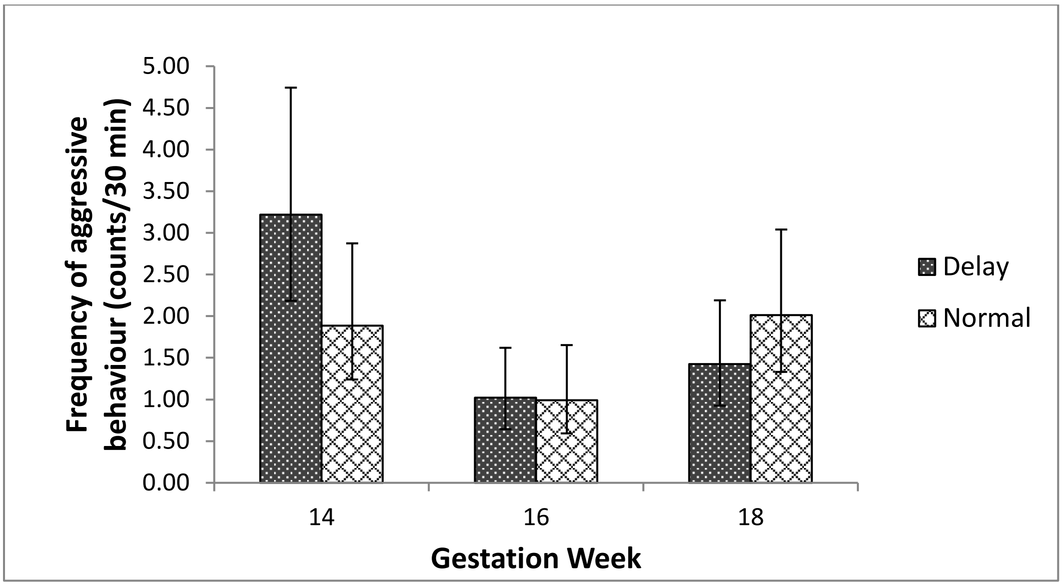

Figure 4.7. Frequency of aggressive behaviour displayed by pregnant ewes from Negative groups according to feeding regime (delay or normal feeding) at the feeding trough in different gestation weeks. Data presented are means of counts/30 min with CI as error bars. Ewes subjected to delayed feeding displayed higher aggressive behaviour than ewes that had been fed normally at week 14 of gestation (P<0.005).

There was no overall effect of treatment on Free Join and Free Leave during concentrate feeding at the feedface. However, there was a significant effect of the interaction between treatment and gestation week in both Free Join and Free Leave (Figure 4.8). From post-hoc tests conducted, Alternative ewes showed a higher proportion of Free Join than Control and Negative ewes at week 14 of gestation ($F_{4,179.3}$ = 2.88, P=0.024) which was the only difference found between treatment groups in the analysis. Post-hoc test also showed that Negative ewes displayed significantly lower Free Join in week 14 compared to week 16 of gestation (Figure 4.8).

As for Free Leave, Control ewes displayed significantly lower proportion of Free Leave in week 14 compared to week 16 and 18 of gestation. Negative ewes also displayed lower proportion of Free Leave before it significantly increased in week 16. But in week 18, the proportion of Free Leave in Negative ewes decreased to the same level as in week 14 of gestation (Figure 4.8; $F_{4,168.2}$ = 4.01, P=0.004).

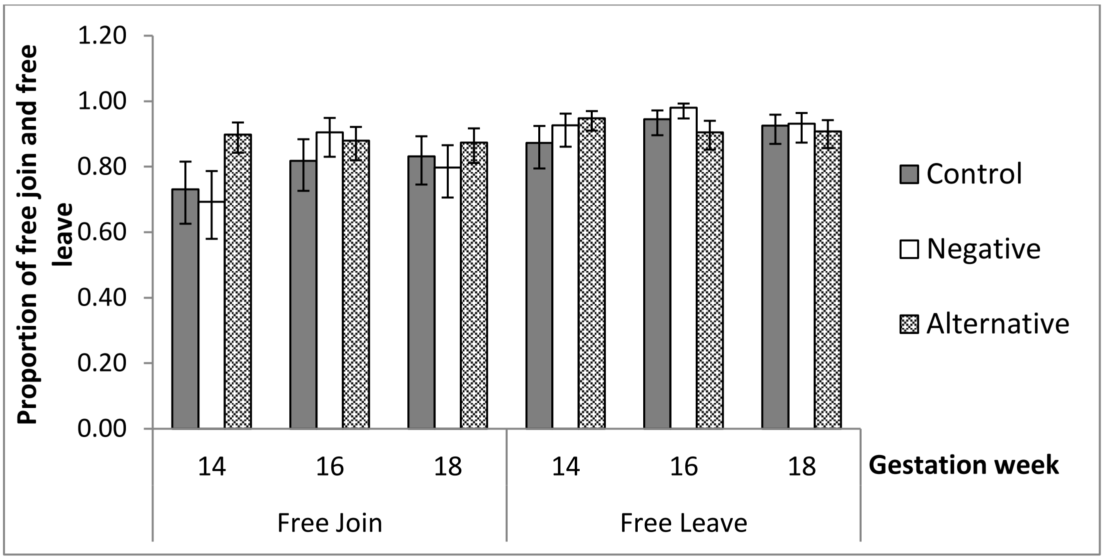

Figure 4.8. Proportion of free join and free leave at different gestation weeks according to treatment group with CI as error bars. At week 14 of gestation, Alternative ewes displayed significantly higher proportion of free join compared to Control and Negative ewes. On the other hand, Negative ewes showed a lower proportion of free join at week 14 compared to week 16 of gestation. As for free leave, Control and Negative ewes displayed significantly lower proportion of free leave in week 14 compared to week 16 of gestation. Results are considered significant at P < 0.005.

There was also a significant interaction between temperament and gestation week (Table 4.4; $F_{4,179.8}$ = 3.07, P=0.018) on the proportion of free join to the feedface. From post-hoc test conducted, high reactivity (HR) ewes showed significantly higher proportion of free join compared to low reactivity (LR) ewes in week 18 of gestation. LR ewes initially displayed a lower proportion of free join in week 14 which significantly increased at week 16 before decreasing at week 18 (Table 4.4). HR ewes also showed a lower proportion of free join initially in week 14 which significantly increased in week 16. No effect of parity was observed in this analysis (Multiparous: 0.83 (0.79-0.87), Primiparous: 0.84 (0.80-0.87); $F_{1,61.9}$ = 0.08, P = 0.774).

Table 4.4. Mean proportion (with CI) of Free Join at the feedface at different gestation weeks according to temperament.

Temperament	Gestation Week			P-value
	14	16	18	
HR	0.76 (0.66-0.83)^{a}	0.87 (0.80-0.92)^{b}	0.90 (0.85-0.94)^{b}	$F_{4,179.8}$ = 3.07, P=0.018
IR	0.83 (0.75-0.89)	0.86 (0.79-0.92)	0.84 (0.76-0.90)	
LR	0.78 (0.68-0.85)^{a}	0.88 (0.81-0.93)^{b}	0.73 (0.64-0.81)^{c}	

^{a,b,c}, means in the same row with different superscripts differ significantly (P<0.05) as determined by post hoc LSD tests.

4.3.4 Faecal glucocorticoid metabolite (FGM)

Ewes from the Alternative group had a significantly higher concentration of FGM compared to Control and Negative groups from week 13 to week 17 of gestation (Figure 4.9; $F_{6,222.0}$ = 14.66, P < 0.001). No difference was found between Control and Negative groups at any gestation week. There was also a significant effect of time (week of gestation) on the concentration of FGM ($F_{3,221.9}$ = 102.29, P < 0.001). Post hoc test showed that, at week 17 of gestation, the concentration of FGM in Control and Negative ewes increased significantly compared to the previous weeks of gestation. For Alternative ewes, the concentration for FGM that was initially low in week 11 of gestation similar to other treatment groups, increased significantly in week 13 and remained high until the end of the experiment. However, no parity (Multiparous: 71.12 (63.82-79.26), Primiparous: 80.35 (72.43-89.14); $F_{1,70.6}$ = 2.65, P = 0.108) and temperament (HR: 79.25 (69.84-89.92), IR: 74.64 (66.08-84.32), LR: 73.11 (64.73-82.59); $F_{2,72.9}$ = 0.46, P = 0.634) effects were found on the concentration of FGM throughout the experiment.

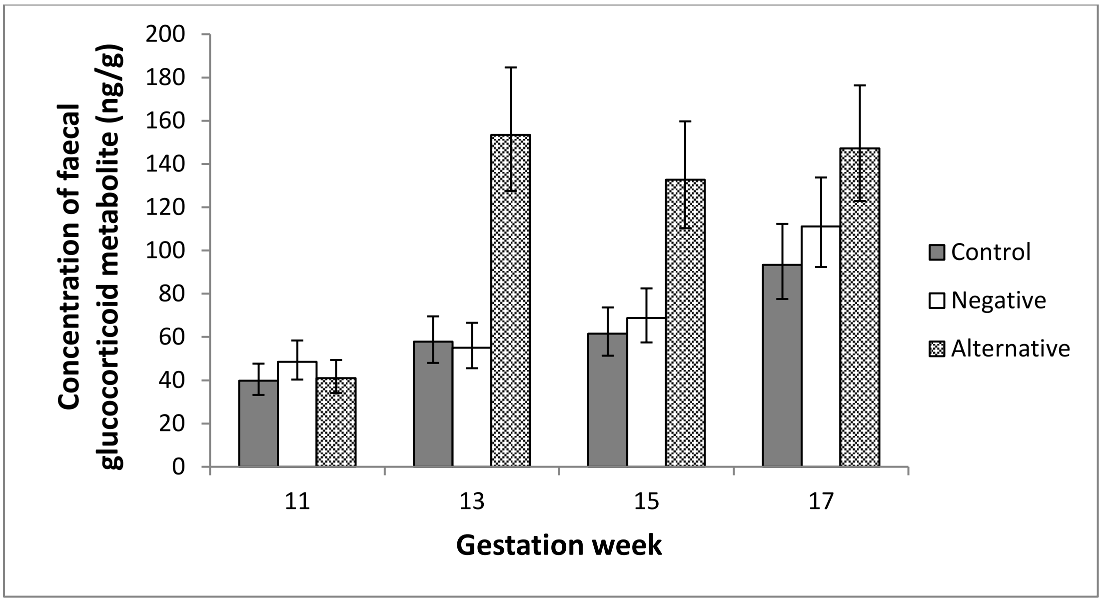

Figure 4.9. Concentration of Faecal Glucocorticoid Metabolite (FGM) in ewes from Control, Negative and Alternative groups at week 11, 13, 15 and 17 of gestation. Data presented are means of FGM concentration with CI as error bars. Alternative ewes had a significantly higher concentration of FGM compared to Control and Negative ewes at week 13, 15 and 17 of gestation (P<0.001).

4.3.5 Haematological parameters

From all the haematological parameters analysed, only red blood cell (RBC), neutrophils (segmented), neutrophil to lymphocyte ratio (NLR) and eosinophil count were significantly affected by treatment group alone (Table 4.5). RBC counts in ewes from the Alternative group were significantly higher than the counts in ewes from Control and Negative groups. Post-hoc analysis on Packed Cell Volume (PCV), which showed a tendency to be affected by treatment, revealed that the Alternative ewes had higher PCV than Control ewes (Table 4.5). Neutrophil count on the other hand was significantly lower in Alternative ewes compared to Control and Negative ewes. In contrast, the eosinophil counts in Negative and Alternative ewes were significantly different from each other but not from the ewes in Control group (Table 4.5).

Table 4.5. Means of haematological parameters (with SEM or CI) in experimental ewes according to treatment groups.

Haematological parameters	Treatment			P-value
	Control	Negative	Alternative	
Red cell parameters				
Red blood cell count ($x10^{12}$/L)	10.45 (0.14)[a]	10.4 (0.14)[a]	10.97 (0.15)[b]	$F_{2,8.7}$ = 4.82, P=0.039
Haemoglobin (g/L)	115.7 (1.4)	116.0 (1.4)	119.6 (1.4)	$F_{2,134.8}$ = 49.37, P=0.153
Mean corpuscular volume (fL)	31.13 (0.23)	31.72 (0.23)	31.53 (0.24)	$F_{2,8.8}$ = 1.69, P=0.239
Packed cell volume (l/l)	0.326 (0.006)[a]	0.33 (0.006)[ab]	0.346 (0.006)[b]	$F_{2,8.6}$ = 3.44, P=0.063
Mean corpuscular haemoglobin concentration (g/L)	357.2 (3.5)	353.1 (3.5)	346.6 (3.5)	$F_{2,8.9}$ = 2.38, P=0.149
White cell parameters				
White blood cell count ($x10^9$/L)	10.12 (0.33)	9.74 (0.33)	9.22 (0.34)	$F_{2,7.90}$ = 1.98, P=0.20
Neutrophils ($x10^9$/L)	3.26 (2.9-3.7)[a]	2.97 (2.6-3.3)[a]	2.5 (2.2-2.8)[b]	$F_{2,8.2}$ = 5.00, P=0.038
Lymphocytes ($x10^9$/L)	6.16 (0.22)	6.03 (0.22)	5.88 (0.23)	$F_{2,7.90}$ = 0.44, P=0.657
Neutrophil to Lymphocyte ratio	0.56 (0.52-0.61)[a]	0.51 (0.47-0.55)[a]	0.44 (0.40-0.47)[b]	$F_{2,6.8}$ = 9.39, P=0.011
Monocytes ($x10^9$/L)	0.17 (0.14-0.20)	0.13 (0.09-0.16)	0.12 (0.09-0.16)	$F_{2,8.2}$ = 2.37, P=0.154
Eosinophils ($x10^9$/L)	1.99 (1.60-2.48)[ab]	1.91 (1.53-2.37)[a]	2.92 (2.36-3.61)[b]	$F_{2,9.0}$ = 4.44, P=0.046
Other parameters				
Platelets ($x10^9$/L)				

	206.54 (173.2-246.3)	212.81 (178.5-253.8)	202.3 (168.9-242.3)	$F_{2,9.0}$ = 0.07, P=0.928

a,b,c, means in the same row with different superscript differ significantly (P<0.05) as determined by post hoc LSD tests.

An effect of gestation week alone was seen in almost all haematological parameters (Table 4.6). RBC count and haemoglobin concentration decreased significantly throughout gestation. In contrast, platelet count significantly increased with gestation week. Mean corpuscular volume (MCV), WBC and neutrophil count were constant in week 13 to week 15 of gestation but increased significantly from week 15 to week 17 of gestation. The opposite was true of the number of eosinophils which decreased from week 15 to week 17. Packed cell volume (PCV) also decreased from week 13 to week 15 and remained constant until week 17 of gestation. As for the mean corpuscular haemoglobin concentration (MCHC), the values differed significantly at all sampling points with the lowest reading at week 13, and the highest at week 15 of gestation.

Table 4.6. Means of haematological parameters (with SEM or CI) in experimental ewes according to gestation week.

Haematological parameters	Gestation week			P-value
	13	15	17	
Red cell parameters				
Red blood cell count ($x10^{12}$/L)	11.36 $(0.1)^a$	10.46 $(0.1)^b$	10.0 $(0.1)^c$	$F_{2,134.7}$ = 103.87, P<0.001
Haemoglobin (g/L)	122.4 $(1.0)^a$	116.5 $(1.0)^b$	112.4 $(1.0)^c$	$F_{2,134.8}$ = 49.37, P<0.001
Mean corpuscular volume (fL)	31.26 $(0.15)^a$	31.15 $(0.15)^a$	31.96 $(0.15)^b$	$F_{2,131.9}$ = 43.44, P<0.001
Packed cell volume (l/l)	0.356 $(0.004)^a$	0.326 $(0.004)^b$	0.320 $(0.004)^b$	$F_{2,134.3}$ = 68.17, P<0.001

Mean corpuscular haemoglobin concentration (g/L)	345.7 (2.2)[a]	358.6 (2.2)[b]	352.6 (2.1)[c]	$F_{2,133.2}$ = 40.83, P<0.001
White cell parameters				
White blood cell count (x10^9/L)	9.24 (0.24)[a]	9.36 (0.24)[a]	10.48 (0.23)[b]	$F_{2,134.4}$ = 16.91, P<0.001
Neutrophils (x10^9/L)	2.34 (2.1-2.6)[a]	2.42 (2.2-2.7)[a]	4.27 (3.9-4.7)[b]	$F_{2,138.6}$ = 79.96, P<0.001
Lymphocytes (x10^9/L)	6.35 (0.17) [a]	6.25 (0.17) [a]	5.48 (0.16) [b]	$F_{2,130.1}$ = 15.21, P<0.001
Neutrophil to Lymphocyte ratio	0.38 (0.35-0.42) [a]	0.40 (0.37-0.44) [a]	0.82 (0.75-0.89) [b]	$F_{2,136.7}$ = 77.47, P<0.001
Monocytes (x10^9/L)	0.05 (0.03-0.09) [a]	0.11 (0.08-0.14) [b]	0.27 (0.23-0.31) [c]	$F_{2,108.8}$ = 16.20, P<0.001
Eosinophils (x10^9/L)	2.86 (2.4-3.4)[a]	2.51 (2.1-3.0)[a]	1.54 (1.3-1.9)[b]	$F_{2,117.4}$ = 11.74, P<0.001
Other parameters				
Platelets (x10^9/L)	171.4 (152.4-192.7)[a]	207.0 (184.1-232.8)[b]	250.61 (222.9-281.8)[c]	$F_{2,134.4}$ = 24.07, P<0.001

[a,b,c,] *means in the same row with different superscript differ significantly (P<0.05).*

The interaction between treatment and gestation week significantly affected lymphocyte count (Figure 4.10; $F_{4,130.2}$ = 2.83, P=0.027). The lymphocyte count in ewes from Control and Alternative groups significantly decreased from week 15 to week 17 of gestation while the count remained constant in Negative ewes from week 13 to week 17 of gestation. Lymphocyte count in Alternative ewes further decreased in week 17 of gestation which made it significantly different from the count in Negative ewes.

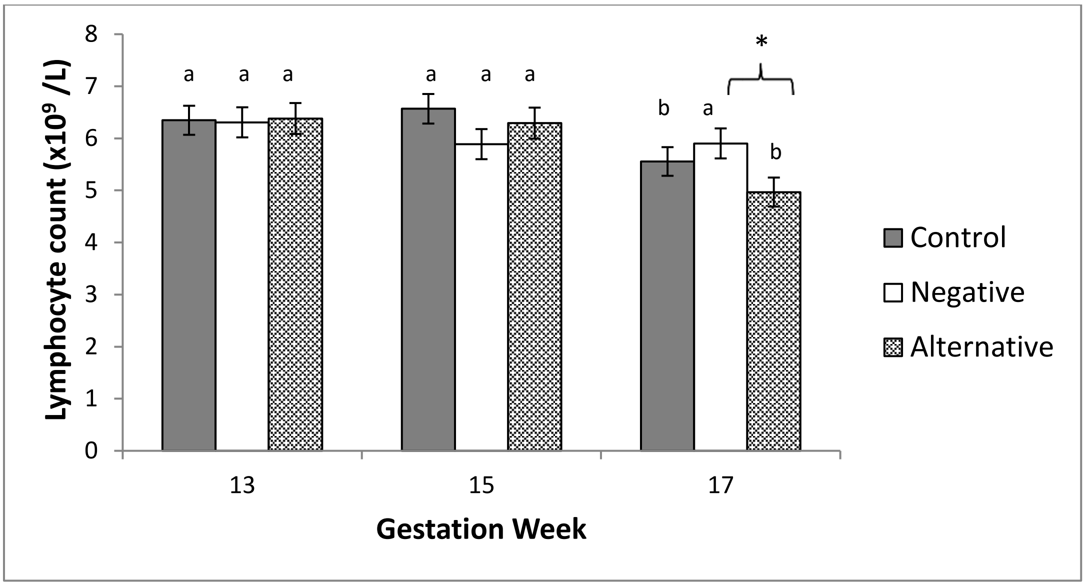

Figure 4.10. Mean lymphocyte count (with SEM as error bars) in ewes from different treatment groups at week 13, 15 and 17 of gestation. Bars with different [ab] superscripts and * indicate significant difference between treatment at different gestation week and between treatment within the same gestation week respectively at P < 0.005.

As for NLR, all groups maintained a constant ratio from week 13 until week 15 of gestation (Figure 4.6; $F_{4,136.8}$ = 0.014, P=0.014). However, NLR increased significantly from week 15 to week 17 of gestation in all three groups. From post-hoc analyses, it was found that ewes in the alternative group had a significantly lower NLR compared to Control ewes in week 13 of gestation and lower NLR than Control and Negative groups in week 15 of gestation (Figure 4.11).

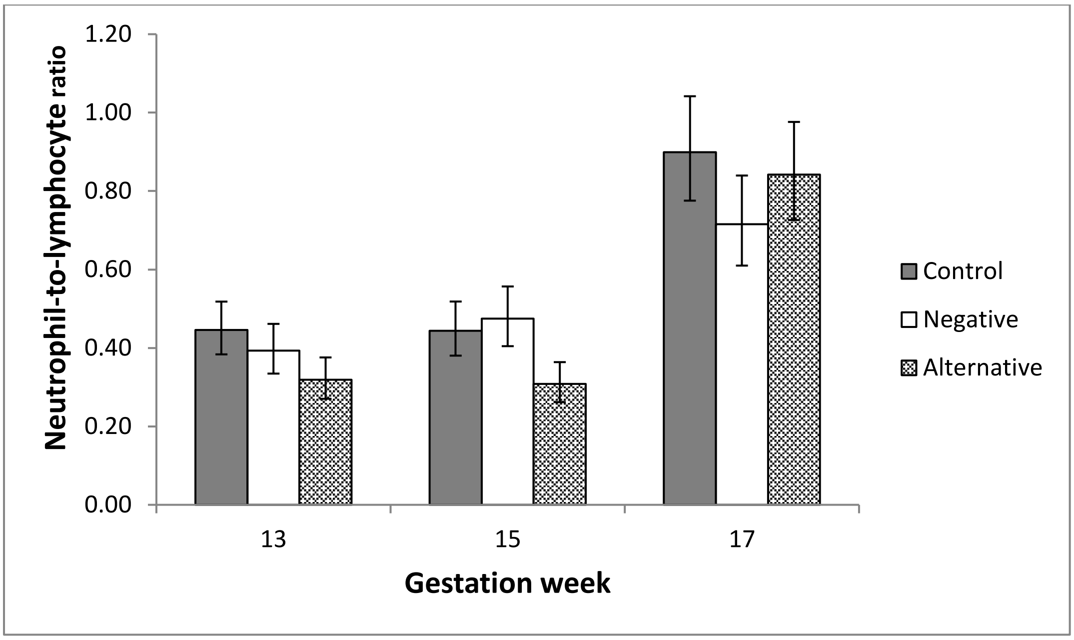

Figure 4.11. Mean NLR (with CI as error bars) in ewes from different treatment groups at week 13, 15 and 17 of gestation. NLR was observed to increase significantly from week 15 to week 17 of gestation in all three groups. Alternative ewes had a significantly lower NLR compared to Control ewes in week 13 of gestation. Data are considered significant at P<0.005.

Eosinophil count was found to be the only blood parameter affected by parity. Eosinophil count recorded in multiparous ewes was significantly higher than the count in primiparous ewes (mean count (CI): Multiparous: 2.67 (2.27-3.15), Primiparous: 1.86 (1.57-2.21); $F_{1,63.4}$ = 11.15, P=0.001).

4.3.6 Oestradiol and progesterone

There was no effect of treatment, parity and temperament on the concentration of oestradiol in the ewes during gestation (Table 4.7).

Table 4.7. Concentration of oestradiol (pg/ml) of pregnant ewes by parity, treatment and temperament.

	Oestradiol (pg/ml)	Confidence Interval	P-value
Parity			
Multiparous	260.02	229.15-295.04	$F_{1,66.8}$ = 0.48, P = 0.490
Primiparous	278.61	246.65-314.72	
Treatment			
Control	278.61	244.43-317.57	$F_{2,7.6}$ = 0.31, P < 0.743
Negative	269.77	238.83-304.73	
Alternative	259.42	228.62-294.36	
Temperament			
HR	260.02	223.03-303.14	$F_{2,68.1}$ = 0.15, P = 0.858
IR	269.15	231.91-312.38	
LR	278.61	237.90-326.29	

[abc] *Within the mean concentration column, different superscripts show significant difference between parity, gestation week or temperament according to post hoc pair comparisons, using Fishers' Unprotected LSD (P<0.05)*

However, week of gestation significantly affected the level of oestradiol in the ewe's plasma. The concentration of oestradiol increased significantly from week 13 to week 15 of gestation (Table 4.8).

Table 4.8. Concentration of oestradiol (pg/ml) of ewes at different gestation weeks.

Gestation week	Oestradiol (pg/ml)	Confidence Interval	P-value
Week 13	237.68[a]	216.78-260.61	$F_{2,139.4}$ = 3.74, P = 0.026
Week 15	279.90[b]	248.91-314.75	
Week 17	292.42[b]	260.04-328.82	

[ab] Within the mean concentration column, different superscripts show significant difference between gestation week according to post hoc pair comparisons, using Fishers' Unprotected LSD (p<0.05)

Progesterone concentration was not affected by treatment group (Control: 47.10 (39.85-55.66), Negative: 38.55 (32.77-45.35), Alternative: 45.71 (38.33-54.51; $F_{2,7.6}$ = 1.69, P = 0.247). However, there was a significant effect of parity where primiparous ewes had higher concentrations of progesterone compared to multiparous ewes throughout the experimental period (Table 4.9). The level of progesterone also significantly increased throughout gestation. There was also a tendency for temperament to affect progesterone concentration. From post-hoc comparison, HR ewes had a significantly lower concentration of progesterone compared to IR and LR ewes (Table 4.9).

Table 4.9. Concentration of progesterone (ng/ml) of ewes by parity, gestation week and temperament.

	Progesterone (ng/ml)	Confidence Interval	P-value
Parity			
Multiparous	39.17[a]	34.06-45.06	$F_{1,155.4}$ = 4.51, P = 0.035
Primiparous	48.53[b]	42.38-55.56	
Gestation week			
Week 13	28.51[a]	25.01-32.50	$F_{2,129.2}$ = 52.5, P < 0.001
Week 15	48.64[b]	42.67-55.44	

Week 17	59.70[c]	52.38-68.05	
Temperament			
HR	36.81[a]	31.01-43.70	$F_{2,156.7} = 3.03$, P = 0.051
IR	48.31[b]	40.88-57.08	
LR	46.45[b]	39.49-54.65	

[abc] Within the mean concentration column, different superscripts show significant difference between parity, gestation week or temperament according to post hoc pair comparisons, using Fishers' Unprotected LSD (p<0.05)

4.4 Discussion

In this study, two housing systems which were designed to represent good and poor housing systems for pregnant ewes, and a Control group representing normal on-farm management in the UK, were established to examine the effect of different housing/feeding systems on the weight, body condition score (BCS), behaviour, physiology and haematology parameters of gestating ewes from mid to late pregnancy. It was hypothesised that ewes from the Negative group would be adversely affected by their housing system while Alternative ewes would show better outcomes in all parameters tested. Surprisingly however, ewes from the Alternative group were found to be the most negatively affected by the different housing condition.

Alternative ewes showed a decrease in BCS and had the lowest weight gain at the end of the experiment compared to other groups. This contradicts the studies conducted by Sormunen-Cristian and Jauhiainen (2001) and Leto et al. (2002) which reported higher weight gain in silage fed ewes during the entire pregnancy compared to ewes fed with hay. Nevertheless, it is worth noting that in both studies, hay and silage fed ewes were also supplemented with concentrate feed. In a study done by Bøe and Andersen (2010), ewes were observed to spend significantly more time feeding on hay instead of silage and displayed higher competition to feed on the hay than in the silage treatment which may indicate greater preference for hay compared to silage. Therefore the low weight gain and BCS in the Alternative ewes may be due to the ewes' preference for roughage as they may find silage to be less palatable. Ideally, food intake and refusals should also be measured to understand the feeding behaviour better though it was difficult to apply to this present study since the ewes were housed in a group. The nutritional requirements of pregnant ewes were calculated prior to this study to ensure the Alternative ewes received a sufficient amount of nutrients throughout pregnancy, although it is possible that all ewes did not consume their entire ration as calculated. Besides the possibility of less palatable silage perceived by the ewes, the texture of the silage may perhaps result in quicker gut fill which slow down the ingestion and rumination process which could then minimize the nutrition intake by the ewes. Primiparous ewes also had a significantly lower BCS compared to multiparous ewes as they may spend less time feeding compared to multiparous ewes which was seen in the first study (Chapter 2). However, general pen behaviour was not recorded for this present study to exactly know if there were difference in time spent feeding between parities.

In a study on food restriction in pregnant ewes, restricted ewes showed greater BCS loss, lower body weight gain and higher concentration of BHOB indicating higher mobilization especially of adipose tissue mass and increased ketogenesis (Bonnet et al., 2015). Interestingly, ewes in Alternative group had the lowest level of beta hydroxybutytrate (BHOB) in plasma compared to Control and Negative groups (although all three groups had normal readings ($\leq$0.7 mmol/l)), which was surprising given their decrease in BCS. However, the reason for this is unknown and therefore will need further study to verify the result. The concentration of faecal glucocorticoid metabolite (FGM) in Alternative ewes was significantly increased from week 11 to 13 of gestation and remained high throughout the experiment. The FGM concentration of the Alternative ewes in week 13 and 15 of gestation were also significantly higher than the ewes in Control and Negative groups which indicate that Alternative ewes might have experienced some sort of stress, either nutritional or psychological, during the experiment.

Surprisingly, this was not supported by the Leukocyte count analysed in the ewes. Stress or glucocorticoid treatment are reported to increase the number of neutrophils and decrease lymphocytes count which eventually will increase the neutrophil-lymphocyte ratio (NLR) (Davis, Maney, & Maerz, 2008). In this study however, Alternative ewes had significantly lower count of neutrophils compared to Control and Negative ewes, while no significant difference was found in the number of lymphocytes in all three groups. The low neutrophil counts also resulted in lower NLR value in Alternative ewes compared to ewes in Control and Negative groups. The cause for this conflicting result is unclear. However there was a possibility that the ewes did not eat sufficient silage to meet requirements which may have eventually caused nutrient deficiency. Deficiency in some nutrients such as copper, folate and iron has been reported to decrease neutrophil count in humans (Dunlap, James, & Hume, 1974; Lazarchick, 2012; Lima et al., 2006; Tamura et al., 1994).

Eosinophil count was also higher in Alternative ewes than in Negative ewes. It is known that high eosinophil count can be caused by parasitic infection (Buddle et al., 1992; Carranza-Rodriguez et al., 2008; Kovalszki & Weller, 2016). Therefore, there is a possibility that the higher count of eosinophil may be due to parasitic infection as it was reported in human studies that nutrient deficiency can increase susceptibility of an individual towards parasitic infection (Anstead et al., 2001; Papier et al., 2014). In fact, research conducted on sheep reported a significant association between body condition score and *Eimeria* infection, an apicomplexan parasite causing coccidiosis disease in animals. Higher infection rate by *Eimeria* was found in sheep with poor BCS compared to sheep with good BCS (Khan et al., 2011), perhaps due to a weakened immune system as a result of nutrient deficiency. Female sheep were also reported to be more susceptible to coccidiosis compared to male as they may be more susceptible to *Eimeria* infection due to physiological stress they had to experience during pregnancy, parturition and lactation (Khan et al., 2011; Khodakaram-Tafti & Hashemnia, 2017; Yakhchali & Zarei, 2008).

On the other hand, in the present study primiparous ewes had significantly lower eosinophil count compared to multiparous ewes. There was a similar finding in dairy cows where the parity difference was only found on eosinophil count but not total leukocytes count and other component of white blood cells (O’Driscoll et al., 2012). The characteristic of leukocytes count can be affected by acute and chronic stress as well as inflammation which can be described by the occurrence of neutrophilia, lymphopenia, eosinopenia and an occasional monocytosis (Cole, Roussel, & Whitney, 1997; Holtenius et al., 2004). This usually occurs in a non-serious condition of inflammation such as dystocia, ketosis, milk fever and indigestion (Cole et al., 1997). Therefore, the lower count of eosinophil found in primiparous ewes may be due to the higher stress experienced in these ewes from being pregnant and giving birth for the first time as well as being exposed to new environmental condition compared to multiparous ewes. Leukocyte profile were also found to change significantly around parturition which are also associated with peripartum stress experienced by animals (Holtenius et al., 2004). At week 17 of gestation, which was the final week of blood sampling before parturition, the haematological parameters showed significant increases in neutrophils and monocytes as well as a significant decrease in eosinophil and lymphocyte counts. These findings were in concordance with the study by Cole et al. (1997) on the characteristics of leukocytes count as a response to stress which has been discussed above. Rats exposed to chronic stress also showed a decrease in total lymphocyte counts (Zager et al., 2007). It is suggested that the significant change in leukocytes parameters found in week 17 of gestation in the present study was due to the higher stress experienced by the ewes at late gestation near to parturition.

As for aggressive behaviour at the feedface, higher aggressive behaviours were displayed by the Negative ewes experiencing delayed feeding compared to during normal feeding on week 14 of gestation which decreased on week 16 and increased again on week 18 of gestation. Increased aggression, activities and movement due to delay and unpredictable feeding have been reported in various animal species such as in primates (Ulyan et al., 2006), cows (Normando et al., 2013) and pigs (Carlstead, 1986). As the concentrate feed was first provided at week 14 of gestation, the higher incidence of aggressive behaviour seen at that week may be due to the heightened anxiety waiting to be fed. They seemed to be habituated by the delayed feed as seen from the lower aggression in the subsequent weeks before increased aggression was observed again in week 18 of gestation which may be due to higher nutritional demand during the final weeks of pregnancy.

Time affected aggressive behaviour displayed by low reactive (LR) ewes when at week 18 of gestation, LR ewes displayed higher aggressive behaviour at the feedface compared to week 14 and week 16 of gestation. This corresponds with the lowest Free Join observed by LR ewes also at week 18 of gestation which implies that LR ewes displayed more forced entry to access the feed. LR ewes in this study were selected for the least activities displayed during the temperament test prior to the start of experiment. However, the least activities displayed might suggest the calm demeanour possessed by these ewes or perhaps they were too afraid that they froze during the test. It is usually assumed that higher level of activity as a stress response indicates higher level of fear when immobility also indicates fear (Boissy & Erhard, 2014) as the animals are too afraid that they froze. In a study conducted on lambs, less active animals in the feedlot (in term of number of steps) had a higher concentration of cortisol (Ric et al., 2016).

In a review, Koolhaas et al. (1999) suggested two distinct behaviours of animals in response to stress which are described as ‘proactive’ responses characterised by higher exploration and aggression as well as ‘reactive’ or withdrawal responses characterised by immobility and low level of aggression. The higher aggression displayed by LR ewes at the feedface in this present study may indicate that they were also experiencing stress although they showed the least activity during temperament test. Caution should be taken in interpreting the results of temperament tests conducted during isolation in relation to subsequent observation conducted in groups as the coping strategies in these two situations might differ.

As for the concentration of oestradiol and progesterone, they showed a gradual increase throughout the experiment as has been shown in previous studies (Bassett et al., 1969; Dwyer et al., 2004). Multiparous ewes had a significantly lower concentration of progesterone compared to primiparous ewes while HR ewes also had lower progesterone level compared to LR and IR ewes. In previous studies, no effect of parity on circulating progesterone of pregnant ewes (Dwyer et al., 2004; Dwyer & Smith, 2008) has been observed and to my knowledge, no study has been conducted to investigate the effect of temperament on circulating progesterone. However, there are breed effects where Suffolk ewes had a significantly higher concentration of progesterone compared to Scottish Blackface ewes (Dwyer et al., 2004). Suffolk ewes are known to be less competent mothers compared to Scottish Blackface (Dwyer & Lawrence, 1998; Dwyer & Smith, 2008; Pickup & Dwyer, 2011) which are similar to the incompetency of primiparous as a mother compared to multiparous ewes (Dwyer & Lawrence, 2000; Dwyer & Smith, 2008). High responsive ewes on the other hand displayed better maternal behaviour at lambing by spending more time with the lambs and less udder refusals when lambs attempted to suck compared to low responsive ewes (Coulon et al., 2014). Prepartum plasma oestradiol, but not progesterone has been reported to be related to individual differences in maternal behaviour (Dwyer et al., 2004). However, very high intra- and inter-plate CV% were found in both oestradiol and progesterone assays in the current study and thus, the results may not be reliable.

In conclusion, this study took a surprising twist when ewes from the Alternative group were the most negatively affected by the different management system in indoor housing even though this group was established to represent a low stress system. The feeding system did not meet the ewes' nutritional needs which may be because of unpalatability, gut fill or some other reasons. This silage was perhaps the main reason that the Alternative ewes displayed symptoms of undernutrition such as decreased body weight and BCS, higher concentration of FGM and altered haematology parameters. There was also a mild effect of parity on the parameters tested such as lower BCS and BHOB in primiparous ewes which were not seen in the first study (Chapter 2) as this may possibly be contributed to the presence of the Alternative group. Thus, this study has shown that undernutrition during gestation may impair the growth, development and physiology of gestating ewes. The effect of these responses to the different housing conditions on the maternal behaviour of ewes will be assessed in the next Chapter.

5. Does an alternative housing system result in better outcomes for ewes and their interactions with their lambs?

5.1 Introduction

In Chapter 4, an attempt was made to reduce the stress experienced by pregnant ewes by allowing continuous access to feed to minimise competition. However, although aggression was reduced, the results obtained were surprisingly not as had been hypothesised as ewes from the Alternative group were found to be the most affected by the housing condition during pregnancy rather than the Negative ewes. It was suggested that the different type of food provided to the Alternative ewes may be the main reason for the altered body weight, body condition score, physiological and haematological parameters in the Alternative ewes compared to Control and Negative ewes during gestation.

Undernutrition experienced by ewes during pregnancy has been shown to result in low lamb birth-weight (Corner et al., 2010; Dwyer et al., 2003; Ferguson et al., 2011; Hammer et al., 2011) and increased lamb mortality (Ferguson et al., 2011; Muñoz et al., 2009; Nordby et al., 1987; Rooke et al., 2010). Undernourished ewes have been shown to spend less time grooming their new-born lambs (Dwyer et al., 2003) and express fewer low-pitched vocalisations than controls (Corner et al., 2010). Ewes undernourished during gestation also show reduced colostrum yield and milk production (Banchero et al., 2006; Bizelis et al., 2000). However, the concentration of IgG in ewe colostrum or the lamb serum or plasma after lambing has not been adequately studied. The available studies are inconsistent with either no difference in lamb plasma IgG (Khalaf et al., 1979) or increased concentration of IgG (Hammer et al., 2011) in lambs from an undernourished mother.

Apart from the lower amount of maternal behaviour displayed by undernourished ewes towards their new-born lambs, compared to well fed ewes, reduced lamb vigour can also impair the bonding between ewe and lamb which could affect lamb survival. Lambs from undernourished mothers show delays in accomplishing the behavioural milestones that need to be achieved after parturition such as standing and sucking (Dwyer et al., 2003). This study also reported that a weak attachment was established between undernourished ewes and their lambs.

Studies of ewes subjected to psychological stress during pregnancy produce inconsistent effects on maternal behaviour depending on the different type of stress imposed on the ewes. For example, aversively handled ewes during pregnancy showed increased grooming towards the lambs compared to ewes which had been gently handled (Hild et al., 2011). However, ewes that were exposed to various aversive challenges such as isolation, mixing and transport showed no difference in maternal behaviour during the first 30 minutes after parturition and during a selectivity test at 2 hours post-partum compared to ewes which were not exposed to aversive events (Coulon et al., 2014).

For this present study, it was hypothesised that the ewes which were affected most by the different management and housing system while living indoors during gestation (the Alternative group), may also display impaired maternal behaviour towards their lambs. The concentration of IgG in the colostrum and faecal glucocorticoid metabolite (FGM) measured 12 hours post partum may also be negatively affected by pregnancy treatment.

5.2 Method

5.2.1 Lambing and data collection

This study involved the same 84 ewes that were part of the study as described in Chapter 4. After the end of the data collection and observation on ewes during gestation, the ewes remained in the same pen and were left undisturbed until parturition. The ewes lambed in April 2015 over a period of 23 days. Two observers were present in the shed 24 hours per day to conduct observations and tests, collect samples and assist the ewes where necessary. Once the ewes were seen to exhibit signs of parturition (i.e. detection of fluids), they were moved to a lambing pen located in front of each experimental pen (see Figure 4.1 in Chapter 4). Intervention and assistance towards the ewes were kept to a minimum and only given if the ewe had failed to progress through parturition in a certain period of time, which was: 1 hour after fluids were detected with no parts of the lamb showing, and/or 2 hours after parts of lamb of the lamb were seen at the vulva without any obvious progress being made (Dwyer & Lawrence, 1998). At 30 minutes postpartum, lambs were caught, their navels were dipped in iodine (to prevent infection) and rectal temperature was taken. In the case where the second born lamb (L2) was born within 30 minutes from the first born lamb (L1), application of iodine and measurement of rectal temperature were conducted 30 minutes post-partum of the second lamb. A coloured tape was looped around the right hind leg of L1 to allow identification of birth order for further observations and tests. When the lamb had dried, the tape loop was replaced by spraying the ewe number on both sides of the lamb's body and a bar over the shoulder (for L1) and rump (for L2). After 2 hours in the lambing pen, each ewe and its lambs were moved into a colostrum collection pen ($1m^2$) where a colostrum sample was collected for further analysis (see 5.2.3). The mother and its lamb were then left in the pen for another four hours to bond before they were then moved to a postpartum pen with other ewes and their lambs.

5.2.2 Observation during parturition

As has been described in Chapter 3, birth was defined as the moment when the pelvis of the lamb had passed through the vulva. During lambing, the assistance given to the ewe/lamb and the birth presentation of the lamb were scored according to the scoring system in Table 3.1 (Chapter 3). The position of the ewe at lambing (whether standing or lying) and whether the ewe lambed during the day or night were also recorded. For this study, 0610 until 2019 hr was considered as day while 2020 until 0609 was considered as night. A High Definition video camera (Canon Legria HFM52, Japan) was placed on a tripod in front of the pen to continuously record the behaviours of both ewe and lamb starting from the birth of L1 up until 2 hours after L2 was born. This was complemented by a continuous 24-hour per day video recording using 14 EZ-Distributors video camera linked to a Geovision digital video-recording system (Australia Pty Ltd) to store and view the footage. Cameras were mounted such that each pen was visible on the video record throughout the lambing period. Vocalisations made by the ewes and both lambs were recorded live using a Psion Workabout handheld computer (Psion PLC, London, UK) for 30 minutes after the birth of L2 (T30), followed by three 10 minutes observations every 20 minutes from 50 minutes after birth until L2 was 2 hours old (T120). The collection of behavioural and vocalisation data (Table 3.2 in Chapter 3) were recorded using The Observer Software (Noldus Information Technology, Netherlands). For the maternal behaviour expressed in the first 2 hours postpartum, behaviour recorded by using the video camera was analysed by The Observer Software (Noldus Information Technology, Netherlands) for data collection. However, in the case where the video recorded was not clear (due to various factors i.e. bad camera angle or observed animals being blocked by other ewes), behaviour recorded using Geovision was used to collect behavioural data using The Observer Software.

5.2.3 Colostrum sampling

Colostrum samples were collected from the ewes two hours after the second lamb was born. The ewes and their lambs were moved from the lambing pen into a 1 m^2 colostrum collection pen to ease the collection process. Colostrum samples were obtained manually from both teats and were then placed in a labelled 5 ml plastic storage tube. All the samples were then frozen at 20^oC immediately after sampling until further analysis of Immunoglobulin G (IgG) concentration.

IgG analyses were carried out using an Ovine IgG ELISA Test Kit (Biopanda Reagents, UK). Details on the analysis method can be found in Chapter 3 (Section 3.2.3). The coefficient of variation (CV%) intra-plates and inter-plates were 9.46% and 19.5% respectively.

5.2.4 Ewe selectivity

At 6 hours post-partum, the ability of the ewes to distinguish their own lamb from an alien lamb was investigated in 40 ewes (Control = 18, Negative = 13, Alternative = 9; Multiparous = 21, Primiparous = 19; HR = 13, IR = 12, LR = 15). Ewes were only tested when there was a suitably-aged lamb at the time of testing to be used as the alien lamb (6-8 hours of age). The ewe and its own lambs were moved from the post partum pen into the test pen ($1m^2$). The lambs were then removed from the ewe and placed in different holding pen out of sight of the test ewe for 3-6 minutes (depending on the order of test). A suitable alien lamb was selected and placed in another pen also for 3-6 minutes. A camcorder mounted on a tripod was placed in front of the pen and positioned such that the whole test pen could be seen. To start the experiment, one lamb (either own (L1 only) or alien lamb (L1 or L2)) was placed in the test pen together with the ewe and the ewe's behaviour (for ethogram see Table 5.1) was observed for 3 minutes. After the first three minutes of observation, the lamb in the pen was taken out and the second lamb was placed together with the ewe and observed for another three minutes. The order of the lamb that was placed first with the experimental ewes was balanced by alternating between the two lambs. The experiment was terminated earlier if the ewe butted the lamb three times or knocked the lamb down once, to prevent injury to the test lamb. The parameters observed in this test were: (a) the difference in behaviour displayed by ewes towards their own lamb or alien lamb (e.g. aggression, acceptance at the udder), (b) duration of the test and (c) frequency of high pitched bleat (HPV) and low pitched bleat (LPV) made by ewes. After the three minutes observation, the alien lamb was returned to its mother, whereas the experimental ewe's own lamb was returned to the holding pen to be with the other sibling until the ewe finished the experiment before they were all returned to their original post-partum pen. Ewes and lambs were checked following reunion with their mothers to ensure that all ewes accepted their own lambs.

Table 5.1. Definition of the ewe's behaviour observed during selectivity test at 6 hour post-partum.

Behaviour	Definition
Accept	Ewe allows the lamb to approach the udder and attempt to suck without moving away.
Reject	Ewe avoids the lamb, does not let lamb reach the udder, may butt, push or show aggression toward the lamb.

Low pitched bleat	Vocalisation made with mouth closed. Rumbling sound.
High pitched bleat	‘Baa’ vocalisation, made with mouth open

5.2.5 Faecal sampling

Faecal samples were collected from the rectum of the ewes at 12 hours post-partum in a small holding pen ($1m^2$). Each sample was placed into a labelled plastic bag, homogenised by hand for ease of processing and then frozen at -20 °C until further analysis. In the lab, faecal samples that had been collected were then extracted and analysed for 11-oxoetiocholanolon cortisol metabolite. The details for sample collection, faecal extraction and enzyme immune-assay (EIA) method were as described in Chapter 2 (section 2.2.5). From the ELISA analysis, the coefficient of variation (CV%) of intra-plates and inter-plates were shown to be 2.54% and 7.79% respectively.

5.2.6 Behavioural observations during lactation

The ewes and their lambs were housed until approximately 3 days after birth (depending on the weather and health of the animals) before being taken to a field (4 ha) where all studied animals were kept as a single flock. Once in the field, the interactions between the ewe and lambs were further investigated when the lambs were between 3 days old until 7 weeks old using instantaneous scan sampling of the whole flock. In addition, focal observations of 35 ewes and their lambs were also recorded when the lambs were between 3 days until 6 weeks old.

Instantaneous scan sampling was conducted on all experimental ewes which still had both of their twins together in the field (n = 74). Besides the one ewe that was removed from this study due to giving birth to triplet (as described in Chapter 4), another nine ewes were also not observed as their lambs were stillborn (n=2), not well and eventually died (n=4), became a pet lamb due to a broken leg (n=1) or were rejected by their mothers (n=2). Scan sampling was used to obtain information on time budgets and ewe-lamb distance as well as distance between the ewes and their nearest neighbour (other ewes). Distances were estimated by taking ewe body length as approximately 1 meter. Scan samples were made once per day (5 days per week) between 1200 and 1500 hour.

For focal observations, a total of 35 ewes were chosen to be observed (Table 5.2). Continuous focal observations on each individual ewe and their lambs were recorded for 15 minutes twice per week between 14:00 and 17:00 by a single observer using Observer data collection software loaded on a Psion Workabout handheld computer (Psion PLC, London, UK). In the focal observations, detailed interactions between the ewe and her lambs, especially sucking interactions, were investigated. The order of observations on each individual ewe was made in a pseudo random manner on every observation day such that each ewe was observed at a different time within the observation period. The ethogram for the behaviours recorded of both ewe and her lamb(s) is given in Table 3.3 (Chapter 3).

Table 5.2. Number of ewes observed during focal observation by parity and temperament in each treatment group.

	Control	Negative	Alternative
Parity			
Multiparous	7	7	6
Primiparous	4	5	6
Temperament			
Low reactivity (LR)	4	5	3
Intermediate reactivity (IR)	5	3	4
High reactivity (HR)	2	4	5

5.2.7 Statistical analysis

In this chapter, the statistical analysis involved the data collected during parturition, maternal behaviour in the first 2 hours postpartum, concentration of IgG in colostrum, behaviours expressed in the selectivity test, concentration of faecal glucocorticoid metabolites (FGM) and the behaviour and spatial relationship between ewes and their lambs on the pastures during lactation. All analyses were conducted using GenStat 16th edition (Hemel Hempstead, UK) software. Data were checked for normality and transformed using log10 or square root transformation when necessary. For all transformed data, the mean are reported together with Confidence Interval (CI) instead of using Standard Error of Mean (SEM) as in untransformed data. Significance was considered to be $p < 0.05$, but some tendencies ($p < 0.1$) are also included. Where significant differences were found, post-hoc comparisons were made using Fishers' Least Square Differences (LSD) tests. Details on statistical analysis for each parameter tested are described below.

5.2.7.1 Parturition data and maternal behaviour

For the analysis in the first 2 hour postpartum, a total of 79 ewes were analysed. Besides the one ewe which had been excluded from analysis in Chapter 4, four more ewes were excluded from the analysis in this chapter; two ewes was not able to be observed due to technical problem with the video, while the other two ewes had a mummified foetus as their second lamb for which times of birth were not able to be determined.

5.2.7.1.1 Parturition data

Assistance given during parturition, presentation of the lamb, birth position and the time of lambing (day or night) were analysed using General Linear Model (GLM) with a binomial function. Treatment group, parity and the interaction between the two were used as fixed effects. Due to a low number of partially assisted ewes, the three scores for assistance given at birth were simplified to only two scores: 0 for no assistance given and 1 which is a combination of lambing manually or assisted partially. A similar approach was applied to presentation of lamb at birth with score 0 for normal presentation and 1 for non-normal presentations. These variables were then used as fixed effects to analyse the maternal behaviour within 2 hr postpartum.

5.2.7.1.2 Maternal behaviour

Maternal behaviour analysis commenced only after the birth of L2 except for the latency to groom the lambs which was analysed after the birth of both L1 and L2. Since factors before and during birth could affect the behaviours of ewes and lambs (Table 5.3), an initial univariate analysis was conducted to identify the important variables to be fitted into the final model. For this purpose, one variable was used at a time with the appropriate response variable. Variables whose p-value ≤ 0.2 were selected to be fitted as fixed effect or covariate along with treatment and parity as an interaction in all multivariate analyses (Table 5.4).

Table 5.3. List of variables used as fixed effect and covariates in the analysis of parturition data and maternal behaviour 2 hours post partum

Fixed effects	Covariates
Treatment	Interval between L1 and L2 birth
Parity	Interval between sign of parturition (fluids seen) and L1 birth.
Time of birth (day or night)	
Assistance given to L1 during birth	
Presentation of L1 during birth	
Ewe position when giving birth to L1	
Ewe position when giving birth to L2	

Circling, forward and backward behaviours were combined into one category (avoidance behaviour) during analysis as they were displayed infrequently. The occurrence of pawing behaviour was also infrequent and therefore, only the percentages from different treatment groups are presented in the results. Pushing and butting (aggressive behaviour) were not analysed as they did not occur at all in the present study.

Table 5.4. Table of response variables analysed 2 hours post partum with the type of analysis, data transformation, fixed effects and covariates.

Response variables	Type of analysis and distribution	Transformation (if any)	Fixed effects and covariate (if any)
Grooming behaviour			
Latency to groom L1	GLM (normal)	Log 10	L1 birth assistance L1 birth position Interval from fluid to L1 birth Time of birth Interval between L1 and L2 birth
Latency to groom L2	GLM (normal)	Log 10	Interval between L1 and L2 birth
Duration of grooming both lambs	Repeated measures	Square root	L1 presentation Interval from fluid to L1 birth Interval between L1 and L2 birth
Sucking behaviour			
Proportion of successful sucked by lamb at T30	GLM (binomial)		L1 presentation L2 assistance L2 presentation Day of birth Interval between L1 and L2 birth

Proportion of successful sucked by lamb at T90	GLM (binomial)		-
Avoidance behaviour			
Avoidance at T30	GLM (binomial)		L2 presentation
Avoidance at T90	GLM (binomial)		L1 birth position L2 birth position
Vocalisation			
Frequency of LPV	GLMM (poisson)		L1 presentation L2 assistance Day of birth
Frequency of HPV	GLMM (poisson)		L2 birth position

5.2.7.2 Concentration of IgG in colostrum

For the concentration of IgG in colostrum (mg/ml), the analysis was run using GLM (normal distribution after Log10 transformation) with treatment, parity, temperament and their interactions were fitted as fixed effect.

5.2.7.3 Ewe selectivity

The data were first analysed on the difference in selectivity test duration between own and alien lambs. The difference in test duration when tested with an alien lamb was then analysed using GLM (normal distribution) after the data were transformed using square root. The fixed effects used in this analysis were treatment, parity, temperament and their interactions. The vocalisations made by the ewes during the test (LPV and HPV) were analysed by using GLMM (poisson distribution) with treatment, parity, temperament and lamb (own or alien) as fixed effects with the individual ewe as a random effect.

5.2.7.4 Concentration of faecal glucocorticoid metabolite (FGM)

Treatment, parity, temperament and their interactions were used as fixed effects in the analysis of the FGM concentration at 12 hours post partum. Plate used during lab analysis was fitted as random effect. Analysis was run using REML Linear Mixed Models function in Genstat.

5.2.7.5 Behavioural observation during lactation

For behaviour of ewes and the lambs observed during scan sampling, only lying, standing, idling, grazing and ruminating behaviour were analysed in ewes due to the infrequent display of other behaviours. Whereas for lambs, only lying, standing, idling, grazing and sucking behaviour were analysed statistically. The data for L1 and L2 were pooled together and averaged before analysis. The analyses of the behaviours recorded during scan sampling were performed using logistic regression in GLMM to model binomial proportion, whereas for ewe-lambs and ewe-ewe distance, the data underwent log 10 transformation and were then analysed using REML Linear Mixed Models.

For the suckling behaviour data collected by focal sampling, the 6 weeks worth of data were averaged over 2 week blocks (week 2, 4 and 6). Frequency of the ewes being suckled by lambs was analysed using GLMM with Poisson distribution while the duration to be suckled was analysed using REML Linear Mixed Model after undergoing Log 10 transformation.

5.3 Results

5.3.1 Parturition

The characteristics of ewes during the process of giving birth to the lambs are shown in Table 5.5. No differences were found between the categorical parameters recorded and the three test factors.

Table 5.5. Parameters recorded during the parturition of both L1 and L2.

Parameters	L1 birth (*n*)	L2 birth (*n*)
N = 80		
Assisted	19	14
Non-normal presentation	16	18
Give birth while lying	67	55
Give birth while standing	13	24
Give birth during the day	49	-
Give birth during the night	31	-

5.3.2 Maternal behavior within 2 hour postpartum

5.3.2.1 Grooming behavior

5.3.2.1.1 Latency to groom L1 and L2 after birth

The latency to groom L1 after birth was not affected by treatment, parity and temperament (Table 5.6; Wald = 3.10, d.f. = 2, P = 0.221). However, after the birth of L2, there was a tendency of the interaction between treatment and parity to affect the latency to groom L2 (Table 5.6; Wald = 5.60, d.f. = 2, P = 0.068). From post-hoc test conducted, it was found that multiparous ewes from Alternative group took significantly longer to start grooming their second-born lambs after birth compared to multiparous ewes from the Negative group. Within the Negative group, primiparous ewes were slower to groom their lambs than multiparous ewes. In addition, ewes with a longer duration between the birth of L1 and L2 were 1.01 (0.99-1.02) seconds quicker to groom L2 compared to ewes which had a shorter duration between births (Wald = 4.81, d.f. = 1, P = 0.032).

Table 5.6. Mean latency (seconds) (with CI) to start grooming L1 and L2 by ewes after the birth of L2.

	Multiparous	Primiparous
L1		
Control	33.65 (15.0-62.17)	55.34 (12.34-137.08)
Negative	42.46 (15.88-113.57)	62.37 (24.73-157.38)
Alternative	95.45 (32.48-280.83)	38.46 (14.64-101.03)
L2		
Control	34.27 (18.55-63.32)^{ab}	54.20 (28.68-102.41)
Negative	24.66 (13.41-45.35)^{a}	80.91 (43.21-151.51)*
Alternative	65.01 (32.0-132.0)^{b}	46.13 (25.08-84.84)

*^{ab} Within the parity column, different superscripts show significant difference between parity while * shows significant difference within treatment according to post hoc pair comparisons, using Fishers' Unprotected LSD (P<0.05)*

5.3.2.1.2 Duration of grooming L1 and L2

There was no effect of treatment, parity and temperament alone on the total duration that ewes groomed their own lambs within two hours after the birth of L2. However, the interaction between treatment and parity affected grooming duration (minute) (Table 5.7; Wald = 12.823, d.f. = 2, P = 0.003). From post-hoc test conducted, multiparous ewes from the Alternative group spent the shortest time grooming their lambs compared to multiparous ewes from the Control and Negative groups. No differences were found in time spent grooming the lambs between all three treatment groups in primiparous ewes. However, post-hoc tests also showed that in the

Alternative group, multiparous ewes spent significantly less time grooming their lambs compared to primiparous Alternative ewes as opposed to the Control group where multiparous ewes spent significantly more time grooming their lambs than primiparous ewes.

Table 5.7. Mean total duration (minute) (with SEM) ewes spent grooming own lambs within two hours after the birth of L2.

	Multiparous	Primiparous
Treatment		
Control	351.6 (15.4)^{a}	296.3 (16.9)*
Negative	343.4 (16.8)^{a}	331.7 (16.1)
Alternative	280.5 (19.0)^{b}	327.6 (16.5)*

*^{ab} Within the parity column, different superscripts indicate significant differences between parity while * shows significant differences within treatment according to post hoc pair comparisons, using Fishers' Unprotected LSD (p<0.05)*

Overall, the ewes also showed a significant decrease in grooming their lambs from the first 10 minutes until 80 minutes after the birth of L2 which then remained constant until 90 minutes of L2 birth (Figure 5.1; Wald = 676.40, d.f. = 5, P < 0.001).

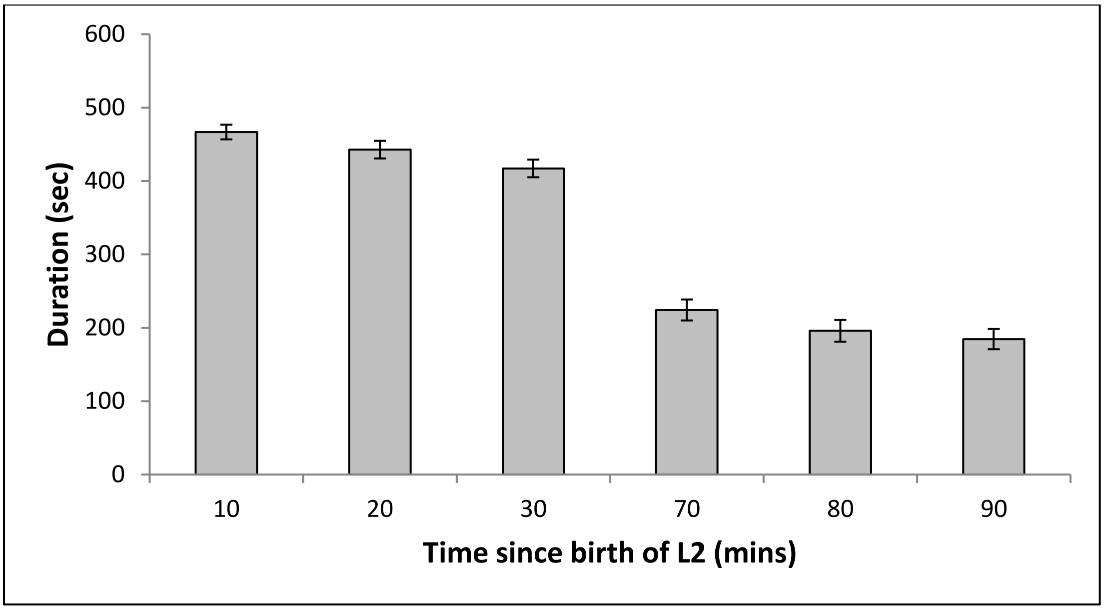

Figure 5.1. Duration of time spent grooming lambs by ewes over the 90 minutes observation period after the birth of Lamb 2 (L2). Observations were conducted in 10 minute windows with a 30 minutes gap between 30 to 70 minutes after the birth of L2. Values are mean duration with SEM. Grooming behaviour decreased significantly from the first 10 minutes until 80 minutes of the birth of L2 ($P<0.001$).

5.3.2.2 Sucking behaviour

During the first 30 minutes after L2 birth, 63.3% of the observed ewes were successfully suckled by their lambs. No treatment, parity or temperament effects were found on the proportion of sucking attempts that were successful (Table 5.8).

However, from all attempts to suck the ewes, L2 lambs with a non-normal presentation during parturition had a higher proportion of sucking attempts that were successful compared to lambs with normal presentation during birth (mean proportion (CI range): Normal presentation: 0.22 (0.19 – 0.26), Non-normal presentation: 0.42 (0.32 – 0.51); Wald= 15.04, d.f.= 1, $P < 0.001$). Ewes which gave birth at night also had a higher proportion of attempted suckles that were successful compared to ewes that gave birth during the day (Day birth: 0.22 (0.19 – 0.27), Night birth: 0.30 (0.25 – 0.36); Wald= 4.97, d.f.= 1, $P = 0.026$).

During 60-90 minutes after the birth of L2, 78.9% ewes had already been suckled by their lambs. There was a treatment effect on the proportion of attempted suckles that were successful from the sucking attempts made by lambs, but no parity and temperament effects were found (Table 5.8). Lambs from both the Negative and Alternative groups had a significantly higher proportion of successful sucks from all attempts made to suck their mother than lambs in the Control group. However, ewes with a shorter interval between the births of L1 and L2 had a significantly higher proportion of suck attempts that were successful compared to the ewes with a longer interval between L1 and L2 birth (0.7 (0.67-0.73); Wald= 9.77, d.f.= 1, P = 0.002).

Table 5.8. Mean proportion (with CI) from all attempts to suck ewes by their lambs which were successful within 30 minutes from the birth of Lamb 2 (L2) and from 60-90 minutes after L2 birth based on treatment, parity and temperament.

	0-30 mins after L2 birth	60-90 mins after L2 birth
Treatment		
Control	0.24 (0.18-0.30)	0.20 (0.17-0.25)
Negative	0.24 (0.19-0.30)	0.37 (0.33-0.42)
Alternative	0.27 (0.22-0.33)	0.35 (0.30-0.40)
	Wald = 0.939, d.f. = 2, P = 0.625	Wald= 31.14, d.f.= 2, P < 0.001
Parity		
Multiparous	0.24 (0.19-0.29)	0.33 (0.29-0.37)
Primiparous	0.26 (0.22-0.31)	0.29 (0.25-0.33)
	Wald = 0.508, d.f. = 1, P = 0.476	Wald = 0.09, d.f. = 1, P = 0.762
Temperament		
HR	0.25 (0.20-0.31)	0.31 (0.26-0.37)
IR	0.27 (0.22-0.33)	0.28 (0.24-0.33)
LR	0.24 (0.19-0.30)	0.33 (0.28-0.38)
	Wald = 0.550, d.f. = 2, P = 0.760	Wald = 0.981, d.f. = 2, P = 0.612

5.3.2.3 Avoidance behaviour

Avoidance behaviour displayed by the ewes towards their lambs when their lambs attempted to suckle during the first 30 minutes after birth was found to be affected only by parity (mean proportion of sucking attempts associated with avoidance (CI range): Multiparous: 0.02 (0.004 – 0.05), Primiparous: 0.09 (0.04 – 0.19); Wald= 23.61, d.f.= 1, $P < 0.001$). Primiparous ewes avoided their lambs more when they reached the udder compared to multiparous ewes. No treatment effect was found to affect the proportion of avoidance behaviour displayed by ewes towards their lambs (mean proportion of sucking attempts associated with avoidance (CI range): Control: 0.02 (0.01 – 0.05), Negative: 0.02 (0.01 – 0.05), Alternative: 0.01 (0.00-0.03) ; Wald= 1.01, d.f.= 2, $P = 0.624$).

Between 60 - 90 minutes after the birth of L2, there was an effect of the interaction between treatment and parity (Wald= 10.0, d.f.= 2, $P = 0.01$). From post-hoc tests conducted, primiparous ewes from the Negative group displayed higher avoidance when their lambs reached udder as compared to multiparous ewes also from Negative group (Table 5.9).

Table 5.9. Mean proportion (with CI) of sucking attempts by lambs that were avoided by the ewes in the 60-90 minutes after birth.

	Multiparous	Primiparous
Treatment		
Control	0.15 (0.05-0.35)	0.09 (0.03-0.26)
Negative	0.03 (0.01-0.12)	0.25 (0.11-0.48)*
Alternative	0.05(0.01-0.21)	0.09 (0.03-0.26)

*Within the treatment row, * shows significant difference between parity according to post hoc pair comparisons, using Fishers' Unprotected LSD (p<0.05)*

5.3.2.4 Aggressive and pawing behaviour

In this present study, no display of pushing and butting behaviour (aggression) were observed in any ewes. As for pawing behaviour, a total of 13 ewes were seen pawing their lambs at both times: 1) During the first 30 minutes after L2 birth and 2) between 60-90 minutes after the birth of L2. Out of the 13 ewes, 53.8% (*n*=7) of pawing behaviour was displayed by ewes from Alternative group, 38.5% (*n*=5) by Negative ewes and only 7.7% (*n*=1) by Control ewes.

5.3.2.5 Ewe vocalisations

Overall, within 2 hours post-partum, ewes demonstrated a significantly higher number of low-pitched vocalisations (LPV) compared to high-pitched vocalisations (HPV) (Median (Interquartile range): LPV: 250.0 (136.0-430.5), HPV: 17 (2.25-89.5); P < 0.001). No effect of treatment, parity and temperament were found on the frequency of LPV (Table 5.10).

Table 5.10. Mean frequency (with CI) of low-pitched vocalisations (LPV) made by the ewes within 2 hours postpartum based on treatment, parity and temperament.

	Frequency (CI)	P-value
Treatment		
Control	98.69 (72.69-133.99)	$F_{2,65.2}$ = 0.24, P = 0.787
Negative	111.05 (80.52-153.15)	
Alternative	113.07 (81.03-157.78)	
Parity		
Multiparous	109.18 (81.69-145.92)	$F_{1,63.2}$ = 0.03, P = 0.856
Primiparous	105.64 (81.08-137.63)	
Temperament		
HR	101.70 (71.89-143.87)	$F_{2,64.4}$ = 0.37, P = 0.691

IR	118.87 (88.07-160.43)
LR	102.51 (74.33-141.38)

However, the interaction between treatment and parity had an effect on the frequency of HPV displayed by the ewes (Table 5.11; $F_{2,79.6}$ = 5.65, P = 0.005). From post-hoc test conducted, multiparous ewes from Alternative group made a significantly lower number of HPV compared to other treatment groups and primiparous ewes from the Alternative group. There was also a tendency for temperament to affect the mean frequency of HPV made by ewes within 2 hours postpartum (Mean frequency (CI): HR: 8.81 (4.33-17.95), IR: 25.03 (13.79-45.41), LR: (5.98-23.48); $F_{2,65.6}$ = 2.86, P = 0.065).

Table 5.11. Mean frequency (with CI) of high-pitched vocalisations (HPV) made by the ewes within 2 hours postpartum.

	Multiparous	Primiparous
Treatment		
Control	19.11 (8.26-44.21)^{a}	16.95 (6.92-41.50)
Negative	33.92 (15.04-76.51)^{a}	11.32 (4.67-27.47)
Alternative	2.4(0.63-9.19)^{b}	22.81 (9.91-52.46)*

*^{ab} Within the parity column, different superscripts show significant difference between treatment group while * shows significant difference within treatment between parity according to post hoc pair comparisons, using Fishers' Unprotected LSD (p<0.05)*

There was also an effect of time on the frequency of LPV and HPV (Table 5.12), when all ewes were considered together. During the first 30 minutes (T30) and between 50 to 120 minutes (T120) after the birth of L2, ewes made significantly more LPV than HPV.

Table 5.12. Mean frequency (CI) of LPV and HPV in 30 minutes at two different time frames, T30 and T120.

	T30	T120	P-value
Low pitch vocalisation (LPV)	127.10 (101.85-158.62)	90.74 (72.29-113.90)	$F_{1,74.5}$ = 32.43, P < 0.001
High pitch vocalisation (HPV)	9.87 (6.44-15.14)	19.20 (12.72-28.98	$F_{1,85.4}$ = 41.88, P < 0.001

5.3.3 IgG in colostrum

There were no significant differences found in the concentration of IgG in colostrum between different treatment groups, parity or temperament (Table 5.13).

Table 5.13. Mean concentration (with CI) of colostrum collected from the ewes at 2 hours postpartum based on treatment, parity and temperament.

	Concentration of IgG (mg/ml) (CI)	Wald test
Treatment		
Control	53.21 (47.11-60.11)	Wald= 3.52, d.f.= 2, P = 0.228
Negative	61.10 (54.09-69.01)	
Alternative	52.60 (46.15-59.96)	
Parity		
Multiparous	55.72 (50.68-61.26)	Wald= 0.02, d.f.= 2, P = 0.886
Primiparous	55.34 (50.33-60.84)	
Temperament		
HR	54.58 (48.75-61.10)	Wald= 2.46, d.f.= 2, P = 0.299
IR	53.09 (47.42-59.43)	

LR	59.16 (53.08-65.92)

5.3.4 Ewe selectivity

At 6 hours post-partum, none of the 40 ewes which underwent the selectivity test displayed any rejection to their own lambs and accepted the lambs completely by staying together for the whole 3 minute observation. However, 42.5% (*n*=17) of the ewes rejected the alien lambs by butting and pushing the alien lambs and therefore 17 of the tests were cut short to avoid further aggression.

The duration of the test was not affected by treatment (mean duration in seconds (CI range): Control: 127.24 (104.48 – 152.24), Negative: 145.68 (117.83-176.50), Alternative: 163.07 (125.48-205.58); Wald= 2.47, d.f.= 2, P = 0.303) and temperament (HR: 151.78 (119.86-187.47), IR: 154.01 (124.07-187.25), LR: 129.73 (104.73-157.41); Wald= 1.73, d.f.= 2, P = 0.430). However, parity was found to significantly affect the duration of the test as multiparous ewes had shorter tests compared to primiparous ewes (Multiparous: 123.43 (101.82 – 147.12), Primiparous: 168.22 (143.29 – 195.15); Wald= 6.64, d.f.= 1, P = 0.014). This was also reflected by the result of chi-square test where only 4 out of 19 (21.1%) primiparous ewes did not complete the 3 minutes test whereas in multiparous ewes, 13 out of 21 (61.9%) did not complete the test (X^2 = 6.812, d.f.= 1, P = 0.009).

During the test, the ewes displayed significantly more high pitch vocalisation (HPV) per minute towards the alien lamb compared to their own lamb regardless of the treatment group, parity or temperament (mean HPV per minute (CI range): Alien lamb: 8.84 (7.44-10.37), Own lamb: 1.00 (0.57-1.56); $F_{1,39.0}$ = 130.94, P < 0.001). The interaction between parity and treatment showed a tendency to affect the rate of HPV during selectivity tests (Table 5.14; $F_{2,32.0}$ = 2.62, P = 0.088). Post-hoc tests revealed that primiparous ewes from the Alternative group made the lowest HPV per minute compared to other treatment groups and their multiparous counterparts.

Table 5.14. Mean rate of HPV per minute (with CI) made by the ewes during the three minutes selectivity test.

	Multiparous	Primiparous
Treatment		
Control	5.81 (4.21-7.67)	3.73 (2.33-5.46)[a]
Negative	4.33 (2.78-6.23)	4.67 (2.92-6.82)[a]
Alternative	4.81(2.62-7.66)[*]	1.33 (0.42-2.76)[b]

[ab] *Within the parity column, different superscripts show significant difference between treatment group while * shows significant difference within treatment between parity according to post hoc pair comparisons, using Fishers' Unprotected LSD (p<0.05)*

There was also a tendency for treatment to affect the rate of low pitch vocalisation (LPV) per minute displayed by the ewes during the selectivity test ($F_{2,34.0}$ = 2.94, P = 0.066). From post-hoc test conducted, Alternative ewes displayed a significantly higher number of LPV compared to Negative ewes regardless of the type of lamb present (Figure 5.2).

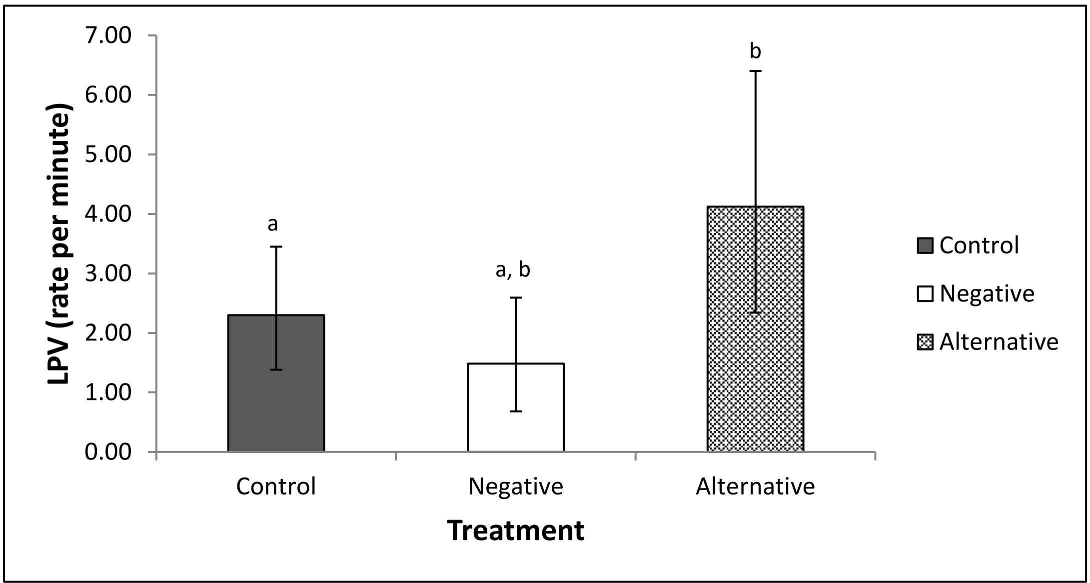

Figure 5.2. Mean number of LPV per minute displayed by ewes within the three minutes of selectivity test from different treatment groups. Error bars are represented by CI. Different superscripts indicate significant difference at P<0.05.

There was also an effect of the interaction between parity and the type of lamb tested, where multiparous ewes made significantly fewer LPV towards the alien lamb compared to their own lamb whereas primiparous ewes made similar numbers of LPV regardless of lamb type (Table 5.15).

Table 5.15. Rate of LPV per minute (means and (CI)) observed within three minutes of selectivity test on their own lamb and alien lamb by different parity.

	Own lamb	Alien lamb	F test
Multiparous	3.68 (2.32-5.36)	0.65 (0.17-1.45)	$F_{1,38.0}$ = 4.45, P = 0.042
Primiparous	4.05 (2.60-5.83)	2.60 (1.46-4.06)	

5.3.5 Faecal glucocorticoid metabolite (FGM)

At 12 hours post-partum, treatment was found to significantly affect the concentration of FGM in the ewe samples (Table 5.16). From post-hoc analysis conducted, Alternative ewes had a significantly higher FGM concentration compared to Control and Negative group. No parity (Multiparous: 194.8 (33.4), Primiparous: 173.8 (33.1); $F_{1,57.5}$ = 0.49, P = 0.488) and temperament (HR: 187.5 (38.4), IR: 193.3 (36.1), LR: 172.1 (34.8); $F_{2,59.0}$ = 0.20, P = 0.823) effects were found to affect the concentration of FGM in ewes.

Table 5.16. Mean concentration (SEM) of faecal glucocorticoid (FGM (ng/ml)) in ewes at 12 hours post-partum based on treatment group.

Treatment group	FGM concentration (ng/ml)	P-value
Control	160.7 (35.3)^{a}	
Negative	145.1 (35.9)^{a}	$F_{2,59.0}$ = 3.34, P = 0.04
Alternative	247.0 (38.9)^{b}	

[ab] Within the FGM concentration column, different superscripts show significant difference between treatment group according to post hoc pair comparisons, using Fishers' Unprotected LSD (P < 0.05).

5.3.6 Behavioural observation during lactation in the field

5.3.6.1 Activity budget

There were no significant effects of treatment or parity on the proportion of observation on all behaviour displayed by ewes during lactation in the field (Table 5.17).

Table 5.17. The proportion of observation where specific behaviours were displayed by ewes observed during scan sampling in the field (standing, lying, walking, idling, grazing and ruminating) by treatment and parity.

Behaviour	Treatment			Parity	
	Control	Negative	Alternative	Multiparous	Primiparous
Standing	0.59 (0.53-0.64)	0.63 (0.57-0.68)	0.63 (0.57-0.69)	0.63 (0.59-0.68)	0.60 (0.55-0.64)
	$F_{2,65.9}$ = 0.63, P = 0.534			$F_{1,64.0}$ = 0.98, P = 0.326	
Lying	0.37 (0.31-0.42)	0.33 (0.27-0.38)	0.30 (0.25-0.37)	0.33 (0.28-0.37)	0.34 (0.29-0.39)
	$F_{2,65.4}$ = 1.19, P = 0.311			$F_{1,63.3}$ = 0.11, P = 0.739	
Walking	0.03 (0.02-0.06)	0.04 (0.02-0.06)	0.05 (0.03-0.08)	0.03 (0.02-0.05)	0.05 (0.03-0.07)
	$F_{2,62.8}$ = 0.91, P = 0.409			$F_{1,63.1}$ = 1.99, P = 0.163	
Idling	0.27 (0.21-0.33)	0.28 (0.22-0.34)	0.26 (0.20-0.32)	0.24 (0.20-0.29)	0.29 (0.24-0.34)
	$F_{2,66.6}$ = 0.12, P = 0.890			$F_{1,65.6}$ = 1.65, P = 0.204	
Grazing	0.41 (0.35-0.48)	0.40 (0.34-0.46)	0.45 (0.38-0.52)	0.44 (0.38-0.50)	0.40 (0.35-0.46)
	$F_{2,65.5}$ = 0.70, P = 0.499			$F_{1,64.3}$ = 0.84, P = 0.363	
Ruminating	0.27 (0.22-0.33)	0.29 (0.23-0.35)	0.25 (0.20-0.31)	0.27 (0.22-0.32)	0.27 (0.22-0.32)
	$F_{2,65.3}$ = 0.39, P = 0.677			$F_{1,63.4}$ = 0.00, P = 0.989	

However, the proportion of standing during observations was significantly affected by the temperament of the ewes ($F_{2,66.5}$ = 4.21, P = 0.019). From post-hoc test conducted, highly reactive (HR) and low reactive (LR) ewes were standing for a higher proportion of observations compared to intermediate reactive (IR) ewes (Table 5.18). There was also a tendency for temperament to affect the proportion of observations where the ewes were grazing ($F_{2,65.7}$ = 3.03, P = 0.055). Post-hoc test conducted showed that HR ewes displayed significantly more grazing behaviour compared to IR ewes (Table 5.18).

Table 5.18. The proportion of standing and grazing behaviour (with confidence interval (CI)) observed by scan sampling during field observations according to the temperament of the ewes.

	Highly reactive (HR)	Intermediate reactive (IR)	Low reactive (LR)
Standing	0.66 (0.61-0.70)^{a}	0.55 (0.49-0.60)^{b}	0.64 (0.58-0.69)^{a}
Lying	0.31 (0.25-0.37)	0.37 (0.32-0.43)	0.32 (0.26-0.38)
Walking	0.03 (0.02-0.05)	0.05 (0.03-0.08)	0.03 (0.02-0.06)
Idling	0.25 (0.20-0.31)	0.28 (0.22-0.34)	0.27 (0.22-0.34)
Grazing	0.49 (0.42-0.56)^{a}	0.37 (0.31-0.43)^{b}	0.41 (0.34-0.47)^{ab}
Ruminating	0.23 (0.17-0.29)	0.30 (0.25-0.37)	0.28 (0.22-0.34)

^{ab} *Within rows, different superscripts show significant difference between temperament group according to post hoc pair comparisons, using Fishers' Unprotected LSD.*

Time after parturition had a significant effect on some behaviours displayed during the field observation (Figure 5.3). Ewes spent a significantly higher proportion of observations idling ($F_{2,389.5}$ = 11.10, P < 0.001) and walking ($F_{2,396.5}$= 3.14, P = 0.05) in the early post-partum period, which then decreased until week 7 post-partum. Ewes also displayed a higher proportion of observations standing during their first three weeks in the field ($F_{2,394.4}$ = 2.39, P = 0.028) which then decreased significantly at week 4 post-partum. However, post-hoc test conducted also showed a significant increased on the proportion of time standing at week 7 post-partum.

In contrast, the proportion of observations when ewes were lying increased significantly from week 1 until week 7 after parturition (Figure 5.3; $F_{2,393.5}$ = 3.59, P = 0.002). Similar trends were also seen in grazing and ruminating behaviour as the ewes displayed significantly less grazing ($F_{2,389.7}$ = 4.80, P < 0.001) and ruminating ($F_{2,392.1}$ = 3.06, P = 0.006) during the earlier weeks of lactation which steadily increased until week 7 after giving birth to the lambs.

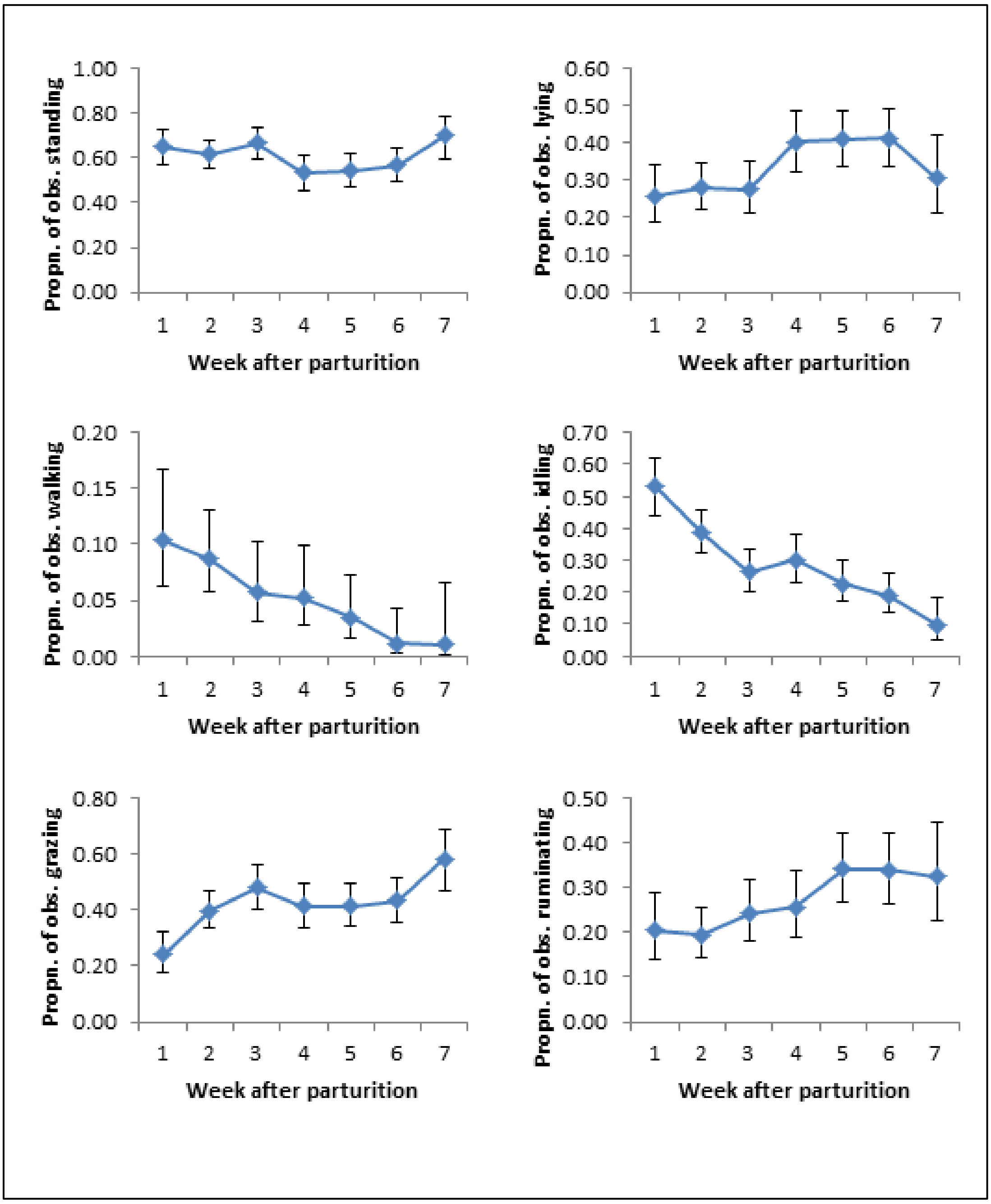

Figure 5.3. The proportion of observation (propn. of obs.) in specific behaviours displayed by ewes observed during scan sampling in the field (standing, lying, walking, idling, grazing and ruminating) at different weeks after parturition. Confidence intervals for each behaviour were expressed as error bars.

As for the lambs' behaviour, no parity and temperament effects were found for any behaviour performed by the lambs (Table 5.19).

Table 5.19. The proportion of observation where specific behaviours were displayed by lambs observed during scan sampling in the field (standing, lying, idling, grazing, sucking) by treatment and parity.

Behaviour	Treatment			Parity	
	Control	Negative	Alternative	Multiparous	Primiparous
Standing	0.35 (0.31-0.40)	0.38 (0.34-0.43)	0.37 (0.33-0.42)	0.39 (0.35-0.43)	0.35 (0.31-0.40)
	$F_{2,442.0}$ = 0.50, P = 0.606			$F_{1,442.0}$ = 1.74, P = 0.188	
Lying	0.61 (0.56-0.66)	0.58 (0.53-0.62)	0.58 (0.53-0.63)	0.58 (0.54-0.62)	0.60 (0.56-0.64)
	$F_{2,442.0}$ = 0.54, P = 0.582			$F_{1,442.0}$ = 0.50, P = 0.480	
Idling	0.81 (0.77-0.84)	0.78 (0.74-0.82)	0.81 (0.77-0.85)	0.80 (0.77-0.84)	0.80 (0.76-0.83)
	$F_{2,442.0}$= 0.53 P = 0.586			$F_{1,442.0}$ = 0.11, P = 0.744	
Grazing	0.01 (0.00-1.00)	0.02 (0.01-1.00)	0.02 (0.01-1.00)	0.02 (0.00-1.00)	0.02 (0.01-1.00)
	$F_{2,60.2}$ = 2.14, P = 0.127			$F_{1,58.8}$ = 2.54, P = 0.116	
Sucking	0.06 (0.04-0.09)	0.05 (0.03-0.08)	0.03 (0.02-0.05)	0.04 (0.02-0.06)	0.06 (0.04-0.08)
	$F_{2,71.1}$ = 2.52, P = 0.088			$F_{1,62.5}$ = 0.53, P = 0.469	

However, there was a tendency for treatment to affect total sucking behaviour in lambs throughout the observation periods ($F_{2,71.1}$ = 2.52, P = 0.088). Post-hoc test revealed that the lambs from the Alternative ewes displayed the least proportion of observations engaged in sucking behaviour which was significantly different from the lambs of Control ewes (Figure 5.4).

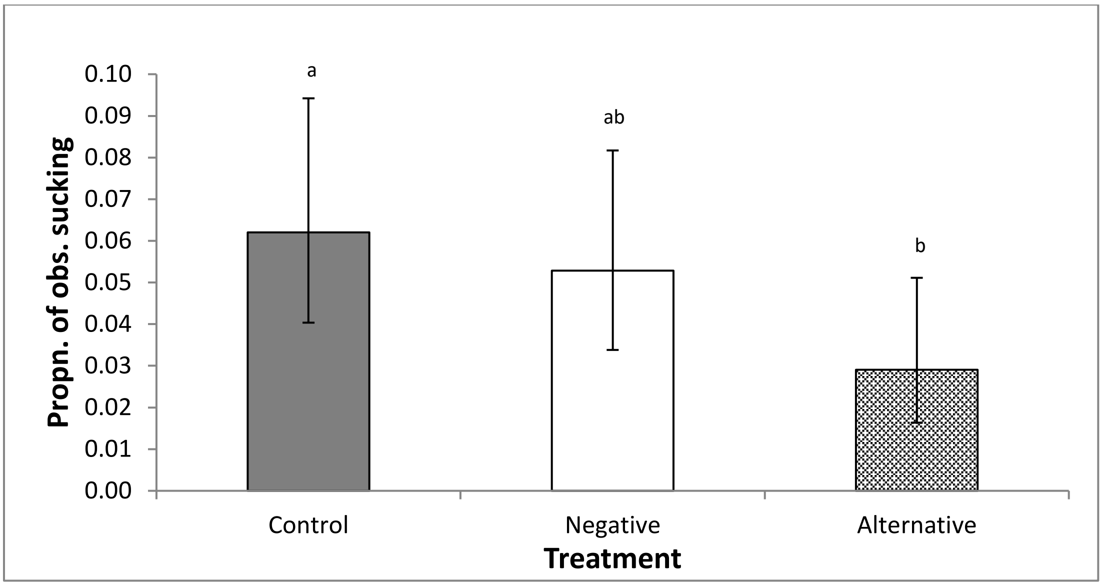

Figure 5.4. The proportion of observation (propn. of obs.) where lambs were sucking as observed from scan sampling conducted during field observations based on the treatment group of the ewes with confidence interval as the error bar. Different superscripts represent significant difference between groups according to post hoc pair comparisons, using Fishers' Unprotected LSD (P < 0.005).

Age of the lamb also had a significant effect on the sucking, idling and grazing behaviour displayed by the lambs (Figure 5.5). The proportion of sucking behaviour was significantly higher when the lambs were in their first week of life which then decreased with time until 7 weeks old ($F_{6,394.3}$ = 7.34, P < 0.001). The same trend was seen in idling behaviour where the proportion of idling decreased from week 1 until week 7 of age ($F_{6,442.0}$ = 7.71, d.f.= 6, P < 0.001). In contrast, the proportion of grazing increased significantly from when the lambs were 1 week until 7 weeks old ($F_{6,399.9}$ = 9.96, P < 0.001).

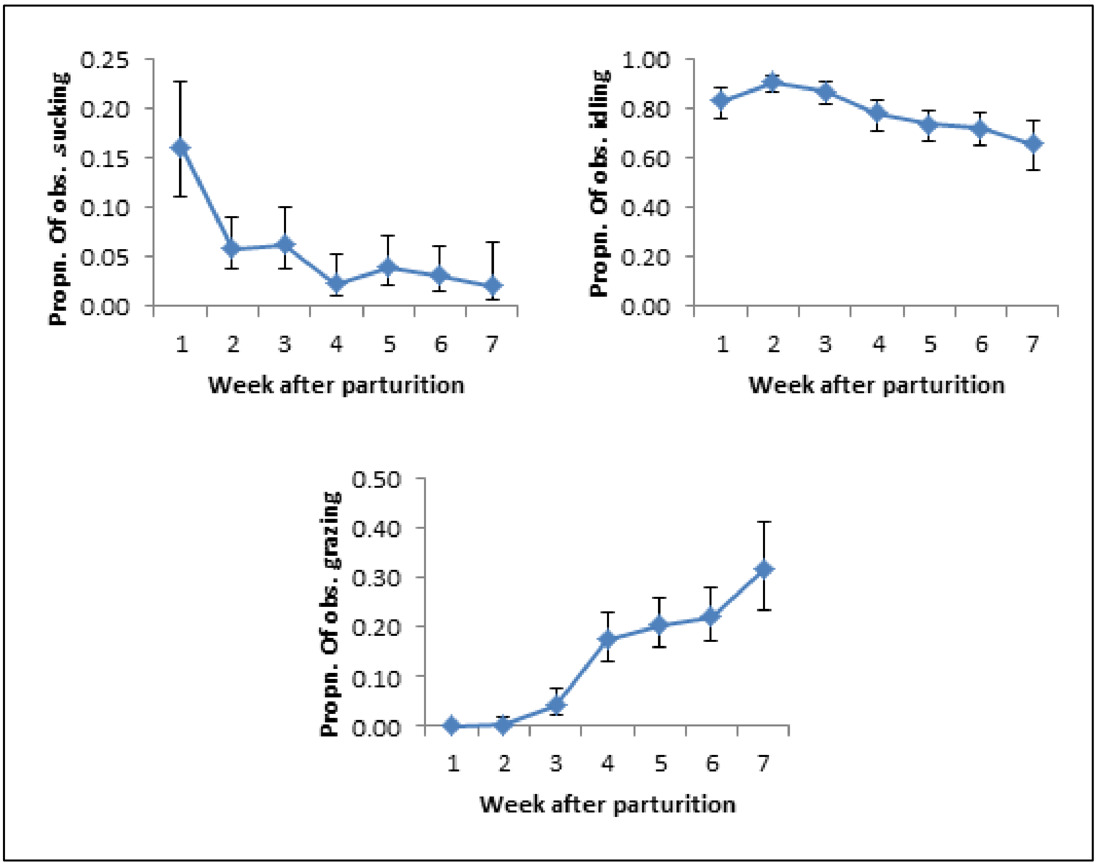

Figure 5.5. The proportion of observations where specific behaviours were displayed by lambs observed during scan sampling in the field (sucking, idling and grazing) at different ages (by week) after parturition. Confidence intervals for each behaviour were expressed as error bars.

5.3.6.2 Spatial relationship between ewes, lambs and nearest neighbour

The distance between ewes and their lambs were significantly affected by the age of the lambs ($F_{6,386.4}$ = 9.22, P < 0.001; Figure 5.6). From post-hoc test conducted, ewes maintained a closer spatial relationship with their own lambs in the first week after birth which significantly increased until week three before it decreased significantly at week four. The distance between the ewes and their lambs then increased again at week five and remained on a plateau until week seven after birth. Treatment, parity and temperament of the ewes did not affect the distance between the ewes and their lambs or neighbouring ewes in any way (Table 5.20).

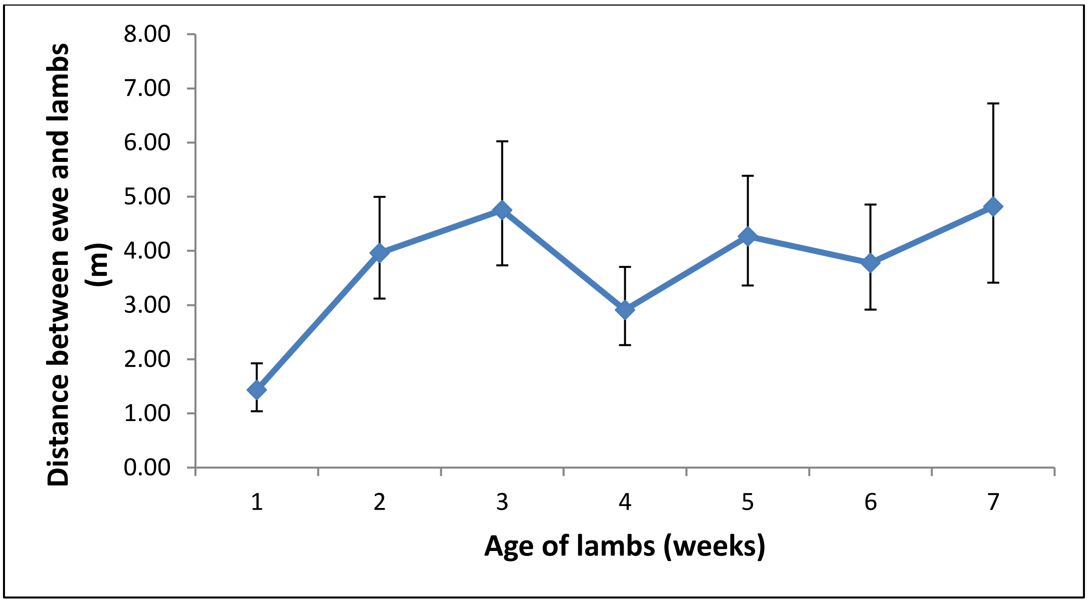

Figure 5.6. Average distance (m) between ewes and their lambs in the field according to the age of the lamb in weeks. The distance between the ewes and their lambs significantly increased from week 1 to week 3 before it decreased significantly at week 4. The distance between the mother and their off-springs increased again at week 5 and remained constant until the end of observation at week 7. Data are significant at P<0.001.

Table 5.20. Treatment, parity and temperament difference in distance (meter) between ewes and their lambs as well as between ewes and their nearest neighbour (other ewe) during focal sampling in the field. Values are means with (CI).

	Ewe-lambs distance (m)	Ewe-neighbour distance (m)
Treatment		
Control	3.82 (3.28-4.46)	4.69 (4.28-5.13)
Negative	4.19 (3.58-4.89)	4.66 (4.25-5.10)
Alternative	4.03 (3.42-4.76)	4.40 (3.99-4.84)
	$F_{2,67.8}$ = 0.34 P = 0.712	$F_{2,68.3}$ = 0.53, P = 0.592
Parity		
Multiparous	4.07 (3.56-4.64)	4.83 (4.31-5.24)
Primiparous	3.96 (3.46-4.53)	4.34 (4.01-4.70)
	$F_{1,65.2}$ = 0.07, P = 0.789	$F_{1,65.8}$ = 3.57, P = 0.063
Temperament		
HR	4.43 (3.74-5.24)	4.75 (4.31-5.24)
IR	4.06 (3.48-4.73)	4.74 (4.33-5.19)
LR	3.59 (3.06-4.22)	4.26 (3.88-4.68)
	$F_{2,66.7}$ = 1.53, P = 0.225	$F_{2,67.2}$= 1.67, P = 0.196

5.3.6.3 Focal sampling on sucking interactions

During focal sampling conducted in the field, 62.9% of ewes at week 1-2, 82.9% of ewes at week 3-4 and 68.6% of ewes at week 5-6 were observed to have been suckled by their lambs. However, there was no effect of treatment, parity or temperament on the occurrence (%) and total duration (seconds) of lambs sucking their mother within the 15 minutes focal observation during lactation in the field (5.21).

Table 5.21. Treatment, parity and temperament difference in sucking behaviour by the lambs during focal sampling in the field. Values are means with (CI).

	Occurrence of sucking (%)	Duration of sucking (s)
Treatment		
Control	81.0 (62.8-91.4)	9.06 (5.08-16.14)
Negative	65.2 (47.2-79.8)	5.91 (3.43-10.20)
Alternative	72.6 (54.6-85.3)	8.09 (4.70-13.93)
	$F_{2,97.0}$ = 0.94 P = 0.395	$F_{2,29.0}$ = 0.61, P = 0.550
Parity		
Multiparous	72.7 (58.5-83.4)	7.64 (5.00-11.71)
Primiparous	74.2 (57.9-85.7)	7.50 (4.57-12.31)
	$F_{1,97.0}$ = 0.02, P = 0.877	$F_{1,29.0}$ = 0.00, P = 0.958
Temperament		
HR	78.3 (59.8-89.8)	8.68 (4.86-15.51)
IR	72.4 (54.2-85.3)	8.63 (5.00-14.90)
LR	69.0 (49.9-83.2)	5.78 (3.28-10.19)
	$F_{2,97.0}$ = 0.32, P = 0.728	$F_{2,29.0}$= 0.65, P = 0.530

5.4 Discussion

In this chapter, the effects of different housing systems, called Alternative and Negative systems, on mother-offspring interactions as well as on the concentration of faecal glucocorticoid metabolite (FGM) post-partum and IgG in colostrum were investigated. It was hypothesised that ewes which were the most negatively affected during gestation by the different housing systems in Chapter 4 would display altered ewe-lamb interactions after parturition.

Treatment alone did not affect grooming behaviour of the ewes towards their lambs. Interestingly however, multiparous ewes from Alternative group took the longest time to groom L2 immediately after parturition and spent the least time grooming L2 compared to Control and Negative ewes as well as when compared to primiparous ewes from the same group. As has been discussed in Chapter 4, Alternative ewes may find silage to be less palatable, or were restricted in their intake in other ways, and therefore, did not eat the ration that would have met their nutritional requirements, which seemed to result in undernutrition of the Alternative ewes during pregnancy. The results obtained were in agreement with previous studies which report that undernourishment in ewes during pregnancy may cause the ewes to take a longer time to interact with their lambs and spend less time grooming their lambs (Dwyer et al., 2003). However, the reason why only grooming behaviour in multiparous ewes was affected is unknown since it has been shown in previous studies that multiparous ewes show a greater quantity and quality of maternal behaviour compared to primiparous ewes (Dwyer, 2008b; Dwyer & Lawrence, 2000).

At 60-90 minutes (T90) after the birth of L2, Negative and Alternative ewes were suckled by their lambs more than Control ewes. It is interesting to note that the stress experienced by both Negative and Alternative ewes might came from different sources which were housing management and undernutrition respectively. Undernourished ewes had been found to suckle their lambs less frequently at 2 hours post-partum (Dwyer et al., 2003) which contradicts the finding observed in the Alternative ewes. However, the increase in sucking frequency by lambs from Negative and Alternative ewes was only observed in T90 instead of from the start of observation at 30 minutes after L2 birth (T30) which may have been caused by a reduction of milk in the udder. Although the milk yield of the experimental ewes was not measured, a higher frequency of sucking by the lambs from Negative and Alternative ewes may be due to the lower production of milk in these two groups compared to Control ewes, leading to a higher sucking frequency in the lambs as they attempt to meet their needs. Some studies in sheep have reported that stress experienced by ewes with regards to housing system may result in lower milk yield and nutrient content produced by the stressed ewes (Finocchiaro et al., 2005; Sevi., et al., 2001b; Sevi, Albenzio et al., 2003; Sevi et al., 1999). Undernutrition in ewes during gestation has also reported to reduce colostrum yield after parturition (Banchero et al., 2006). Interestingly, and of particular relevance to this study, it has also been reported that sheep fed using total mixed rations can reduce milk yield as the sheep may select very little among the dietary ingredients (Pulina et al., 2006).

During 30 minutes after the birth of L2, primiparous ewes displayed a higher proportion of avoidance behaviour compared to multiparous ewes when the lamb attempted to suck. The interaction between parity and treatment was then seen at 60-90 minutes after L2 birth where primiparous ewes from Negative group displayed a higher proportion of avoidance when the lambs reached the udder compared to its multiparous counterpart. One of the reasons may be due to lower milk yield as has been discussed above since there was no treatment difference in the proportion of ewes which avoided sucking attempts 30 minutes after the birth of L2. On the other hand, primiparous ewes have also been shown in some studies to be less cooperative with the lambs sucking attempts (Alexander et al., 1993; Dwyer & Lawrence, 1998, 2005; Dwyer & Smith, 2008) which may be due to the fear and anxiety from the novelty of having lambs for the first time. Based on the concentration of FGM during gestation and 12 hours post-partum, the housing system to which Negative ewes were subjected was not impactful enough to activate the HPA axis unlike the higher concentration of FGM recorded for Alternative ewes. However, prolonged exposure to stress may return the circulating levels of corticosteroid hormones to baseline as part of adaptation mechanisms (allostasis) (Korte et al., 2005; Mormède et al., 2007). Animals with allostasis are more susceptible to anxiety disorders (Korte et al., 2005) which may explained the higher avoidance displayed by primiparous ewes from Negative group in this study when the lambs reached the udder to attempt to suck.

Multiparous ewes from the Alternative group displayed significantly fewer high-pitched vocalisations (HPV) during 2 hour post-partum compared to other ewes. HPV is considered as a protest or distress bleat as opposed to low-pitched vocalisation or rumble (LPV) which is made almost exclusively to the lamb to increase the ewe-lamb bond (Dwyer et al., 1998). It is worth noting that the low rate of HPV was displayed by the same ewes which took the longer time and spent the least time grooming their lambs after the birth of L2. Besides, Alternative ewes also had the highest concentration of FGM during gestation and 12 hours after parturition. Therefore, the low HPV made by these ewes may not be because they have experienced the least stress but due to chronic stress they experienced during the experiment. In humans, some types of chronic stress were found to produce withdrawal and disengagement behaviour as a coping strategy (Gold & Chrousos, 2002; Mason et al., 2001). These studies on the chronic stress in human produced a similar result as in a previous study on sheep, where undernourished ewes were observed to be less attached to their lambs compared to adequately fed ewes (Dwyer et al., 2003) which may best explain the results found in this study with regard to Alternative group. However, why multiparous ewes from Alternative group were the most negatively affected is unclear and further studies should be conducted to clarify whether the result was just an artefact or a genuine finding.

During the selectivity test conducted at 6 hours post-partum, Alternative ewes made significantly more LPV regardless of the lamb present (own or alien lamb) compared to Control and Negative ewes. Since there was no treatment difference in the acceptance or rejection of own or alien lambs during the test, it is difficult to conclude whether the maternal behaviour of the Alternative ewes have been negatively impaired with a sudden increased maternal behaviour compared to at 2 hour post-partum or the results may have occurred by chance. Multiparous ewes were observed to display higher rejection towards the alien lamb compared to primiparous ewes, which shortened the duration of the selectivity test. This suggests that primiparous ewes were more accepting of alien lambs and still not able to fully distinguish between their own lamb and alien lamb even at a close distance at 6 hours postpartum. This was supported by the LPV displayed during the test where primiparous ewes showed no difference in the frequency of LPV towards their own and alien lamb unlike multiparous ewes where the frequency of LPV displayed when tested with alien lamb was significantly lower compared to when tested with their own lamb. It is known that, as in other animals, ewes giving birth for the first time may not be as competent as the more experienced ewes in caring for their young which may result in higher offspring mortality (Dwyer & Lawrence, 2005). Primiparous ewes display higher behavioural disturbance towards the lamb, but after parturition, they show as much total grooming and LPV as more experienced ewes (Dwyer & Lawrence, 2000) which is in agreement with the results found in this present study. The sensitive period for ewes to develop olfactory recognition of their own lamb may only be present for the first 30 to 60 minute after parturition which is at a similar rate to multiparous ewes (Keller et al., 2003). (Poindron & Neindre, 1980) showed in their study that 2 to 4 hours of mother-offspring contact in sheep are necessary and enough for the ewes to recognize their lambs at suckling. The failure to establish selectivity with their own lambs during this time window may result in rejection of their own offspring (Dwyer, 2007, 2014; Dwyer et al., 1998). Results obtained in this study on maternal behaviour displayed by the ewes 2 hour post-partum showed no parity difference for maternal behaviour displayed by the ewes 2 hour post-partum (except for higher avoidance during sucking attempt by lamb which has been discussed above) which suggested that both multiparous and primiparous ewes may have successfully established an attachment with their own lambs. However primiparous ewes may not yet have extended this to rejection of an alien lamb since primiparous ewes displayed as much LPV to the alien lamb as to their own lamb and showed the least rejection to the alien lamb in the selectivity test 6 hour post-partum which implies that primiparous ewes may not distinguish between lambs even at close quarters. This suggests that at 6 hours after parturition, primiparous ewes may have not fully developed selectivity to their own lambs.

Treatment was found to affect suckling behaviour during the observation in the field by instantaneous scan sampling. Alternative ewes were the least likely to suckle their lambs during the 7 week field observation regardless of the age of the lambs. This is similar to a previous study on lambs from undernourished mothers which have been reported to suck less frequently albeit at 2 hours post-partum (Dwyer et al., 2003). The same study also observed less attachment between undernourished ewes and their lambs compared to ewes which were fed normally and this suggests that undernutrition experienced during gestation may impair the mother-offspring interaction in sheep.

The concentration of IgG in this study was not affected by treatment, parity or temperament of the ewes. Studies conducted by Hashemi et al. (2008) and Khalaf et al. (1979) on undernourished ewes also showed no difference in the concentration of IgG in colostrum regardless of nutritional status during gestation. However, in previous studies, increases in concentration of IgG after lambing was recorded in undernourished ewes during gestation although the total colostral IgG was reduced (Swanson et al., 2008). Hammer et al. (2011) also reported an increase in serum IgG concentration in lambs from restricted feeding ewes although the mechanism for the increase is still unknown.

Effects of temperament were only seen on the standing and grazing behaviour during field observation with high reactive (HR) and low reactive (LR) ewes spending a higher proportion of time standing compared to intermediate reactive (IR) ewes, and HR ewes spending a significantly higher proportion of time grazing than IR ewes. In previous studies, less active (labelled calm) ewes were recorded to display higher frequency of grooming and bleating to their lambs than more active ewes (labelled nervous) (Murphy et al., 1994). Calm ewes have also been reported to have a higher concentration of IgG in their colostrum compared to nervous ewes (Hart et al., 2009). However, no temperament effect was found to have an impact on maternal behaviour in this study.

During the 7 week observation, ewe-lamb distance increased from week 1 until week 7 except on week 4 where there was a sudden decrease in the distance between ewe and lambs. This may have happened as the ewes and lambs were introduced to a new pasture during the 4th week of observation and therefore stayed close to their lambs to cope with the anxiety due to the novelty of the pasture. Generally, close proximity between ewe and her lamb may not only indicate maternal protection from predators (Hewson & Verkaik, 1981), but could also facilitate the sucking interactions between the mother and off-spring as well as establishing foraging patterns in the lamb ((Black-Rubio, Cibils, & Gould, 2007). In the first 2 weeks after parturition, ewes are responsible for maintaining the proximity with their lambs and this changes over time where after a few weeks, lamb assumes the primary responsibility to find its dam when they become separated (Arnold & Grassia, 1985; Pickup & Dwyer, 2011).

In conclusion, the interaction between Alternative ewes and their lambs were negatively impaired as had been hypothesised. They displayed behaviours which had been reported as indicators of undernourishment such as taking longer to begin grooming their lambs, shorter grooming bouts and were less cooperative when their lambs attempted to suck. The concentration of faecal glucocorticoid metabolite in Alternative ewes was also significantly higher than Control and Negative ewes, which implied they were experiencing stress. However, no effect of treatment was seen on the concentration of IgG in colostrum, or on the establishment of selectivity, which was influenced only by parity. Special attention must be paid in providing pregnant ewes with high quality and sufficient feed, which is not only being beneficial to the welfare of the ewes, but also the welfare of the lambs.

6. General Discussion and Conclusion

6.1 Introduction

The majority of lamb mortality during the neonatal period usually occurs within 1-3 days after parturition (Nowak et al., 2000). The high mortality not only contributes to the low profitability of sheep farming worldwide but may also bring about negative impacts towards animal welfare in sheep production (Binns et al., 2002). As has been mentioned in Chapter 1, lamb mortality from immediately after birth until approximately 1 week old is usually due to the mismothering-starvation complex and hypothermia as a result of adverse weather condition, low intake of colostrum as well as behaviour impairment of the ewe or her lamb (Kuchel & Lindsay, 1999; Nowak & Poindron, 2006). Postnatal mortality has also been reported to increase in larger flocks (>900 ewes) where they might have less supervision (Binns et al., 2002). Therefore, as one of the steps to reduce lamb mortality, ewes are often housed indoors during late pregnancy and at lambing and this allows the farmer to have better supervision and have greater control of nutrition and health of the pregnant ewes which could be beneficial to the welfare of the animals.

The indoor housing system, which is usually related to the social and physical environment of farmed animals, may be perceived as a stressor to some individual animals (Braastad, 1998). Animals may be kept in inappropriately sized groups or with unsuitable conspecifics, or may also be subjected to mixing and regrouping once or more as part of their husbandry management (Rutherford et al., 2012). In a survey conducted on commercial sheep farms in the UK, nearly half of farms were reported to house pregnant ewes indoor prior to lambing and mixed the ewes into a new social group at least once during housing (DEFRA AW0509, 2013), which may act as stressors for the animals. However, the effect of inadequate housing systems on the pregnant ewes and how it could affect maternal behaviour postpartum are not well studied. Since the survival of the lambs is highly dependent on the maternal behaviour displayed by the ewes as well as the behaviour of the lambs themselves (Madani et al., 2013; Matheson et al., 2012), it is important to eliminate any risk during indoor housing that could affect maternal behaviour.

The main objective of this thesis was to investigate whether indoor housing systems experienced by pregnant ewes may affect the expression of maternal behaviour towards their lambs postpartum. Maternal behaviour was hypothesised to be negatively affected as a result of chronic stress, which they might have experienced during pregnancy from the setting and management of the indoor housing. In this chapter, the summary of the main findings, the limitations and benefits of the studies will be discussed. Recommendations for further study and for the management of pregnant ewes will also be presented throughout the discussion.

6.2 Summary of main findings

The first study (Chapter 2) was designed to replicate commercial practices in the way pregnant ewes are kept in indoor housing. RS-Mix ewes, which were subjected to space and feedface allowances of 1.27 m^2 and 36 cm^2 per ewe respectively (half the space allowance allocated to Control ewes) as well as being subjected to social mixing, were not found to be as disturbed by the housing system as it was hypothesised. The effect of treatment in this chapter was only seen in the display of aggressive behaviour and ruminating behaviour. RS-Mix ewes displayed significantly greater aggressive behaviour during concentrate feeding at week 14 of gestation and higher rumination at the final week of observation (week 18 of gestation) compared to Control group. It was actually parity, which was balanced in all pens, that had the greatest impact on ewe weight and physiology. Primiparous ewes had a lower weight gain at the first two weighing session and had higher concentration of faecal glucocorticoid metabolites (FGM) compared to multiparous ewes. Although there was little effect of treatment on the parameters tested in the ewes with no difference in FGM concentration found between Control and RS-Mix ewes, aggressive behaviour was also observed in the Control ewes during concentrate feeding and this led to the establishment of an alternative system to further minimize aggressive behaviour (Chapter 4).

In the second study (Chapter 4), as mentioned above, an Alternative housing system was set up with the aim to eliminate as many stressors as possible while being housed indoors. These ewes were provided with a much bigger space and feedface allowance than recommended by DEFRA. They were also provided with ad libitum grass silage which was sprinkled with vitamin and minerals as well as high quality protein made from soya. This was done in order to minimize aggressive behaviour they might display if they were provided with concentrate feed once or twice per day as observed in Chapter 2.

A Negative group was also established which had a similar space and feedface allowance as the Control group, but the ewes in the Negative group were subjected to unpredictable and delayed concentrate feeding as well as being exposed to the presence of dog 6 times throughout the experiment. These treatments were considered to be within the normal range of events that sheep might experience during housing. It is worth noting that the space and feedface allowance for the Control and Negative groups in Chapter 4 were similar to the RS-Mix group instead of the Control group in Chapter 2. In the Chapter 4 study, all the pens were balanced for the parity and also temperament of the ewes.

Surprisingly, Alternative ewes were the most negatively affected by the housing management and system allocated to them. The low weight gain and low body condition score (BCS) suggested the possibility of undernutrition as the main reason for the alteration since Alternative ewes were provided with different type of food (ad libitum grass silage) from Control and Negative ewe (ad libitum hay and concentrate feed). The concentration of FGM was also the highest in the Alternative ewes at all sampling points which suggests that Alternative ewes may have experienced chronic stress due to undernutrition. This may result in Alternative ewes being in a more catabolic state metabolically and therefore releasing glucocorticoids to increase access to metabolites. Ewes from the Alternative group also had different outcomes in the haematological parameters compared to Control and Negative ewes. Primiparous ewes in this study had lower BCS throughout the experiment and also had the greatest reduction in BCS from week 13 to 19 of gestation compared to multiparous ewes.

As for the effect of housing systems conducted in Chapter 2 on the maternal behaviour postpartum (Chapter 3), RS-Mix ewes displayed higher avoidance behaviour when their lambs attempted to suck within 2 hours after parturition compared to Control ewes despite very little evidence of chronic stress observed in these ewes during pregnancy. The primiparous ewes from RS-Mix group were also slower to approach their own lamb during a recognition test at 12 hours postpartum compared to other groups.

Despite showing some indicators of experiencing stress during pregnancy (Chapter 4), maternal behaviour displayed by Alternative ewes did not differ to Control and Negative ewes (Chapter 5). The only effect of treatment alone was recorded in the concentration of FGM where, at 12 hours postpartum, Alternative ewes had the highest FGM concentration compared to Control and Negative ewes. However, Alternative and Negative ewes also had the highest proportion of being successfully suckled from the sucking attempts made by their lambs compared to Control ewes. In a selectivity test at 6 hours postpartum, Alternative ewes displayed the highest frequency of low-pitched vocalisation (LPV) regardless of whether their own lamb or an alien lamb that was present during the test.

The interaction between treatment and parity also showed some unexpected outcomes. Multiparous ewes from the Alternative group were slower to begin grooming and spent the least time grooming their lambs after the birth of L2 compared to their primiparous counterpart.

6.3 Does indoor housing system and management during pregnancy results in chronic stress to pregnant ewes?

From the results in Chapter 2, one can easily make a conclusion that the ewes may not be experiencing chronic stress throughout the experiment since RS-Mix ewes were only observed to be different from Control ewes in the higher aggression displayed during the first week when they were provided with concentrate feeds. However, the higher proportion of avoidance with lambs sucking attempt and longer time taken to approach their own lamb during a recognition test, which was observed in the RS-Mix ewes after parturition, indicated that the housing treatment may have had an impact on the ewes that was not detected in the measures made during pregnancy.

Stress generally has been known to increase the concentration of glucocorticoids and catecholamines in sheep (Dwyer & Bornett, 2004). However, as mentioned before no difference in the concentration of FGM was found between RS-Mix and Control ewes. One of the reasons for the lack of disparity in FGM concentration between Control and RS-Mix ewes may have been due to the resistance or down-regulation of the glucocorticoid receptor (GR). The occurrence of hypocortisolism in response to chronic stress has been well documented (Hannibal & Bishop, 2014). It was also suggested that chronic mild stressors may promote a dysthymic-like state (Anisman & Merali, 1997). In human, dysthymia, which can be characterised by a prolonged period of depressed mood, is not accompanied by elevated cortisol levels unlike in major depression patients (Anisman & Matheson, 2005). The cortisol concentration in people with dysthymia may also actually reduce which suggest a down regulation of hypothalamic-pituitary-adrenal (HPA) axis (Griffiths et al., 2000). Similar observation was observed in posttraumatic stress disorder (PTSD) patients, such as combat veterans and Holocaust survivors, where they had reduced cortisol secretion (Yehuda et al., 1995; Yehuda et al., 1995).

The ability of an organism to cope with stressors using behavioural means is one of the important factors influencing neurochemical effects of stressors (Anisman & Matheson, 2005). The coping strategies and methods may vary across individuals, situations and the type of stressor (Carver, Scheier, & Weintraub, 1989). As observed in this present study, RS-Mix ewes spent more time ruminating during the final week of the experiment compared to Control ewes. It has been suggested that rumination might act as a coping mechanism in chronically stressed sheep (Dwyer & Bornett, 2004). During confrontation with stress, an adaptive coping response allows glucocorticoids to return to baseline levels. However, the coping could also be maladaptive where for example, in repeated exposure to acute stress or chronic stress, the excessive or prolonged cortisol secretion could cause cortisol dysfunction, which results in hypocortisolism (Hannibal & Bishop, 2014). Miller et al. (2007) on the other hand suggested that chronic stress does both: increases and decreases HPA activity but at different times during exposure to the stressors. After cortisol begins to increase shortly after exposure to a stressor as a result of an activated HPA axis, in the course of time, cortisol output may reduce to below normal as the body mounts a counter-regulatory response. This may happen as HPA axis is regulated by a negative feedback circuit where the increase in the concentration of cortisol can suppress corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) output (Miller et al., 2007).

The lack of difference in FGM between treatment groups also may have happened as both treatment groups may have experienced stress. As the first sampling of faeces for FGM analysis was conducted about five days after the ewes were housed in the experimental shed, glucocorticoid production in all ewes may have been affected by the movement indoors from pasture to the housing system. Increased plasma cortisol was found when moving sheep indoors from pasture which then took several weeks to normalise (McNatty & Young, 1973; Pearson & Mellor, 1976). It is therefore recommended to collect the faecal sample of the ewes during the first instance they were gathered from the field to be housed indoor in order to get the baseline concentration of FGM.

Since FGM concentration did not differ between treatment groups during pregnancy, the mechanism that caused the impairment of maternal behaviour in RS-Mix ewes is therefore unclear. As this present study was aimed to replicate the condition in some commercial farms, where ewes are exposed to a number of putative stressors, the exact stressor which may have led to the impairment in maternal behaviour is also difficult to be determined. Further studies may be needed to investigate the effect of each housing stressor on the display of maternal behaviour by the ewes towards their lambs. The space allowance for the ewes in this study was set to exceed the minimum space recommended by DEFRA (Department of Environment Food & Agriculture, 2002). If future studies found the same alteration of maternal behaviour as found in RS-Mix ewes, the minimum space allowance recommended by DEFRA might need to be revised.

6.4 *Why would a mother show more or less maternal care if she is stressed?*

As has been mentioned in previous chapters, the few studies which have been conducted in sheep investigating the effect of stress during pregnancy on maternal behaviour postpartum had produced inconsistent results depending on the type of stressors and species. In sheep, no difference was found in maternal behaviour score between yarding, shearing and Control ewes during mid pregnancy (Corner et al., 2010) while higher grooming behaviour was displayed by aversively treated ewes compared to ewes being handled gently during gestation (Hild et al., 2011). In a different study, ewes that were exposed to various aversive events such as social isolation, mixing and transport showed no difference in maternal behaviour to the Control ewes during the first 30 minutes after parturition and during a selectivity test at 2 hours postpartum (Coulon et al., 2014). However, when being separated from their lambs during a maternal motivational test at 48 hours postpartum, stressed ewes vocalised less than control ewes.

Different farms utilize different lambing systems, although the most commonly used system in the UK is once-a-year lambing. This is because in temperate regions, as the sheep only come into oestrus during a relatively short period of time, ewes are usually mated with the ram in winter to ensure that they lamb in spring when grass growth should provide the best nutrition for their offspring. The frequency of having offspring, the number of litters produced and the length of gestation may contribute to the different strategies in animals for how much energy should be invested in caring for their young. From an evolutionary point of view, animals should produce as many viable offspring as possible. As sheep in the UK can only have one or two offspring per year and undergo relatively long gestation period (approximately 145-150 days), the ewes may provide higher maternal investment in keeping each individual offspring alive even in challenging times or conditions. This may perhaps be the reason that the maternal behaviour observed in this study by the ewes exposed to putative suboptimal housing during gestation (RS-Mix, Negative and Alternative ewes) was not as bad as hypothesised though some impairment could still be observed. This is different to the strategies of animals which give birth to a large litter of relatively small offspring, such as in mice, where they have a short gestation period and each litter consists of 6-12 young that are able to reproduce at approximately 30 days of age. Unlike in sheep, there are fairly consistent reports where impaired maternal behaviour was observed in rodents exposed to stress during pregnancy (Baker et al., 2008; Champagne & Meaney, 2006; Klaus, Schöpper, & Huber, 2013; Moore & Power, 1986; Patin et al., 2002). Perhaps from the point of view of rodents, it is not worth it to highly invest energy into maternal care during bad conditions as even by losing a few pups, they are still leaving genes into the next generation. The rodents might adopt this strategy as a trade-off where they invest less on the current offspring as they can produce more in a fairly short time. As generation intervals are long and the litter size is low, it would make evolutionary sense for ewes to invest heavily in their young regardless of the environmental conditions.

6.5 Parity does matter

Overall, as has been observed in this study, primiparous ewes were the most negatively affected by the indoor housing system during pregnancy in term of body weight, BCS, behavioural, physiological and haematological parameters. They have also been shown to be less competent mothers than multiparous ewes postpartum. These impairments in maternal behaviour may be due to their younger age, higher anxiety as well as greater neophobia due to novel conditions experienced by primiparous ewes. Besides being pregnant for the first time, it was also the first time they were being kept indoors for quite a long period and had to experience the limited space in the pen as well as exposure to a new type of food and social grouping. In addition, this was also the first time they have given birth and have interacted with offspring. Novelty has long been recognized to elicit fear or stress responses in animals (Désiré et al., 2004).

In the present study, the primiparous ewes were all two years old whereas multiparous ewes were all three years old. Although they may be closer to their mature size and weight, the two year old ewes may still need some time to complete their own growth (Dwyer & Lawrence, 2000). They may have directed more energy for their own growth which resulted in lower weight gain and reduced body condition as can be observed in primiparous ewes in this study.

Therefore, a one-size-fits-all approach may not be applicable to all animals when managing a farm since there is individual variation of the animals. This is different to the practice of most farms currently where sheep are generally managed as a whole flock instead of as individual animals. It is of course impossible to consider the temperament of each individual ewe when keeping animals indoors. However, as inexperienced animals have been shown to have different responses towards a wide range of novelty compared to more experienced animals (Dwyer, 2014; Van Reenen et al., 2002), parity should be taken into account when designing the system and management plan for indoor housing. For example, primiparous ewes could be gradually introduced to indoor housing by moving them to a pasture right next to the shed where the sheep are exposed to living in pens starting with only a few hours per day which increases over time. At the same time, they should also be gradually exposed to the type of food that will be provided during their stay in the pen. The primiparous ewes may also be kept together without mixing them with unfamiliar multiparous ewes during their first pregnancy. As demonstrated in this study (Chapter 2), multiparous ewes displayed higher aggressive behaviour during concentrate feed and this may discourage the readily anxious primiparous ewes from feeding at the feed trough together with multiparous ewes. Lamb mortality in primiparous ewes has been reported to be higher compared to multiparous ewes (Alexander et al., 1993). Therefore, in terms of sheep farming, applying some of these recommendations might be helpful in minimizing the loss the farmer may have had from having primiparous ewes on the farm.

6.6 Conclusions

In conclusion, suboptimal housing conditions experienced by ewes during pregnancy could mildly impair maternal behaviour postpartum even though only minor evidences of chronic stress were observed in the ewes during pregnancy in the present studies. It is therefore important to be extra careful in making a judgement about the condition of housing system based only on the behaviour and physiological parameters of the ewes during pregnancy. The lack of behaviour and physiological parameters during pregnancy indicating stress may perhaps be due to an adaptive coping strategy by the ewes or maladaptive response which results in cortisol dysfunction. Although impairment of maternal behaviour could be observed in the two main studies in this thesis, it was not as bad as initially expected since the ewes may have invested highly in maternal care to ensure the survival of their offspring. Special attention should not only be given to the management of housing system during pregnancy, but extra measures should also be taken in managing primiparous ewes in ensuring their good welfare in indoor housing which could minimise the loss of their lambs as well as having economic benefits for sheep producers.

7. References

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